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U.S. PATENT APPLICATION

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Invention: MODIFIED CARBOHYDRATE PROCESSING ENZYME

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SPECIFICATION

MODIFIED CARBOHYDRATE PROCESSING ENZYME

Field of Invention

The invention relates to modified carbohydrate processing enzymes and their use in the hydrolysis of glycoside substrates and the synthesis of glycosides.

Background to the Invention

Recent advances in the development of carbohydrate based therapeutics (Koeller and Wong, *Nat. Biotechnol.*, **18** (2000) 835-841), and the limitations of present chemical synthetic methods for producing oligosaccharides, has led to more novel approaches to the synthesis of carbohydrates and their conjugates (Davis, *J. Chem. Soc. Perkin Trans.*, **1** (2000) 2137). One approach to this problem is to carry out such syntheses using carbohydrate processing enzymes such as glycosyltransferases or glycosidases, as a valuable source of catalytic activity for the manipulation of unprotected carbohydrates (Crout and Vic, *Curr. Opin. Chem. Biol.*, **2** (1998) 98-111); Wymer and Toone, *Curr. Opin. Chem. Biol.*, **4** (2000) 110-119; Watt *et al.*, *Curr. Opin. Chem. Biol.*, 7 (1997) 652-660; Kren and Thiem, *Chem. Soc. Rev.*, **26** (1997) 463-473; and Palcic, *Curr. Opin. Biotechnol.*, **10** (1999) 616-624). Glycosidases are simple, robust, soluble enzymes, and in general have been preferred for such glycosynthesis (Scigelova *et al.*, *J. Mol. Catal. B Enzym.*, **6** (1999) 483-494 and Van Rantwijk *et al.*, *J. Mol. Catal. B Enzym.*, **6** (1999) 511-532). Although catalysis of the hydrolysis of glycoside bonds is normally observed, glycosidases may be successfully used to synthesise glycosides through reverse hydrolysis (thermodynamic control) or transglycosylation (kinetic control with activated donors) strategies.

Thus far, improvements in glycosidase synthetic utility have largely focused upon developing new strategies for increasing low product yields (Mackenzie *et al.*, *J. Am. Chem. Soc.*, **120** (1998) 5583-5584), improving regioselectivity of transfer (Prade *et al.*, *Carbohydr. Res.*, **305** (1998) 371-381) or characterising available glycosidases for novel activities (Scigelova *et al.*, *supra*). For example, a major advance in improving yields has been the development of the glycosynthase by Withers and co-workers (Mackenzie *et al.*, *supra*; Mayer *et al.*, *FEBS Lett.*, **466**

(2000) 40-44; Malet and Planas, *FEBS Lett.*, **440** (1998) 208-212; Moracci *et al.*, *Biochemistry* 37 (1998) 17262-17270; Trincone and Perugino, *Bioorg. Med. Chem. Lett.*, **10** (2000) 365-368; Fort *et al.*, *J. Am. Chem. Soc.*, **122** (2000) 5429-5437; and Nashiru *et al.*, *Chem. Int. Ed.*, **40** (2001) 417-420). These nucleophile-less glycosidase mutants are capable of glycosyl transfer in yields of up to 90% using glycosyl fluoride donors, but do not hydrolyse glycoside products and they illustrate well the benefits of glycosidase engineering for creating more synthetically useful catalysts.

An area of glycosidase engineering which has thus far been largely neglected is the engineering of new substrate specificities (Zhang *et al.*, *Proc. Natl. Acad. Sci. USA.*, **94** (1997) 4504-4509; Andrews *et al.*, *J. Biol. Chem.*, **275** (2000) 23027-23033; Kaper *et al.*, *Biochemistry* **39** (2000) 4963-4970; and Rye and Withers, *Curr. Opin. Chem. Biol.*, 4 (2000) 573-580). Since the nature of the parent carbohydrate to be coupled to a given acceptor may be determined in synthesis simply through appropriate choice of donor, it is largely the stereoselectivity of a given glycosidase that we wish to exploit. An area of growing interest is that of combinatorial biocatalysis: the use of enzyme catalysts in parallel reactions to provide arrays of related molecules (Michels *et al.*, *Trends Biotechnol.*, **16** (1998) 210-215; and Krstenansky and Khmelnitsky, *Bioorg. Med. Chem.*, 7 (1999) 2157-2162). In particular, the importance of gaining access to diverse arrays of glycoconjugates has recently been highlighted (Barton *et al.*, *Nat. Struct. Biol.*, **8** (2001) 545-551). However, although combinatorial chemistry has revolutionised the approach to traditional chemical synthesis, the development of combinatorial biocatalysis has been hampered by the often stringent substrate specificities of synthetically useful enzymes.

Summary of the Invention

The present invention provides a polypeptide having carbohydrate processing enzymatic activity, said polypeptide comprising an amino acid sequence selected from:

(a) the amino acid sequence of SEQ ID NO:2 comprising a mutation in at least one of W433, E432 or M439;

(b) the amino acid sequence of a family 1 glycosyl hydrolase, comprising at least one mutation at an amino acid residue equivalent to W433, E432 or M439 of SEQ ID NO: 2; and

(c) a variant of (a) or (b) having carbohydrate processing enzymatic activity and comprising at least one amino acid mutation at a position equivalent to W433, E432 or M439 of SEQ ID NO: 2.

The present invention also provides for the use of a polypeptide of the invention in a method for:

(a) hydrolysis of one or more β-glycosides;

(b) glycoside synthesis of one or more β-glycosides; and/or

(c) transglycosylation of a molecule.

The mutation is preferably a substitution of one of the above-identified amino acid residues with a cysteine (C) residue. The cysteine may be chemically modified so as to alter the electrostatic or steric environment within the active site and thereby alter the enzyme specificity.

The present invention further provides: a polynucleotide encoding a polypeptide of the invention; a vector comprising a polynucleotide of the invention; and a host cell transformed with a polynucleotide or vector of the invention.

Brief Description of the Figures

Figure 1: Partial sequence alignment of the -1 binding pocket motif of Sulfolobus solfataricus β-glycosidase (SSβG) (Cubellis *et al.*, *supra*) with high sequence similarity (left hand column gives SWISSPROT or TrEMBL annotation, numbering is that of SSβG); glycosidases with similar substrate specificity (a) to SSβG and glycosidases with different and/or broadened specificities in which E432 (d), W433 (c) and M439 (b, c, d) differ (marked with arrow and highlighted) (Dalbergia cochinchinensis β-glucosidase - Cairns *et al.*, TREMBL Accession No. Q9SPK3; *Costus speciosus* furostanol-β-glycoside hydrolase - Inoue *et al.*, *FEBS Lett.* **389** (1996) 273-277; LPH_HUMAN, human lactase phlorizin hydrolase - Mantei *et al.*, *EMBO J.*, 7 (1988) 2705-2713; MY3_SINAL, myrosinase from *Sinapsis alba* - Xue *et al.*, *Plant Mol. Biol.*, **18** (1992) 387-398; LACG_STAAU (6-

PBG), *S. aureus* 6-phosphogalactosidase - Breidt and Stewart, *Appl. Environ. Microbiol.*, 53 (1987) 969-973).

Figure 2: Overall activity of chemically modified mutant enzymes (CMMs) with *p*NPGal relative to wild-type (WT) (average over 3 runs, except * average over 2 runs) with standard deviation error bars.

Figure 3: Overall activity of chemically modified mutant enzymes (CMMs) with *o*NPGalP6 relative to wild-type (WT) (average over 3 runs, except * average over 2 runs) with standard deviation error bars.

Brief Description of the Sequences

SEQ ID No 1 provides the amino acid sequence of the β-galactosidase of *Sulfolobus solfataricus* as well as the encoding polynucleotide sequence.

SEQ ID No 2 provides the amino acid sequence of the β-galactosidase of *Sulfolobus solfataricus*.

SEQ ID No 3 provides the amino acid sequence of the –1 binding pocket motif of the β-galactosidase of *Sulfolobus shibatae.*

SEQ ID No 4 provides the amino acid sequence of the –1 binding pocket motif of the β-galactosidase of *Sulfolobus acidocaldarius.*

SEQ ID No 5 provides the amino acid sequence of the –1 binding pocket motif of the β-galactosidase of *Thermoplasma volcanium.*

SEQ ID No 6 provides the amino acid sequence of the –1 binding pocket motif of the β-galactosidase of *Pyrococcus furiosus*.

SEQ ID No 7 provides the amino acid sequence of the –1 binding pocket motif of the β-glycosidase of *Agrobacterium tumefaciens*.

SEQ ID No 8 provides the amino acid sequence of the –1 binding pocket motif of the β-D-glucoside glucohydrolase of *Bacillus circulans.*

SEQ ID No 9 provides the amino acid sequence of the –1 binding pocket motif of the β-D-glucoside glucohydrolase of *Agrobacterium sp.*

SEQ ID No 10 provides the amino acid sequence of the –1 binding pocket motif of the β-glucoside of *Rhizobium meliloti.*

SEQ ID No 11 provides the amino acid sequence of the –1 binding pocket motif of the β-glucoside of *Bacillus halodurans.*

SEQ ID No 12 provides the amino acid sequence of the –1 binding pocket motif of the β-D-glucoside glucohydrolase of *Paenibacillus polymyxa.*

SEQ ID No 13 provides the amino acid sequence of the –1 binding pocket motif of the β-galactosidase glucohydrolase of *Pyrococcus woesi.*

SEQ ID No 14 provides the amino acid sequence of the –1 binding pocket motif of the β-glucoside of *Dalbergia cochinchinensis.*

SEQ ID No 15 provides the amino acid sequence of the –1 binding pocket motif of the Furostanol β- glucoside of *Costus specious.*

SEQ ID No 16 provides the amino acid sequence of the –1 binding pocket motif of the Lactase phlorizin hydrolase of *Homo sapiens.*

SEQ ID No 17 provides the amino acid sequence of the –1 binding pocket motif of the Myrosinase of *Sinapis alba.*

SEQ ID No 18 provides the amino acid sequence of the –1 binding pocket motif of the 6-phospho-beta-galactosidase of *Staphylcoccus aureus.*

SEQ ID Nos 19 to 23 provide the nucleotide sequence of various oligonucleotide primers.

Detailed Description of the Invention

The present invention provides a modified carbohydrate processing enzyme which shows an altered substrate specificity compared to the unmodified enzyme. Preferably, the alteration in substrate specificity leads to the enzyme accepting a broader range of substrates than the unmodified form.

The modified carbohydrate processing enzymes of the invention are typically produced by modifying a family 1 glycosyl hydrolase. In a preferred embodiment, the family 1 glycosyl hydrolase may be one isolated or originating from a thermophilic organism. For example, the enzyme may be from the thermophilic microbe *Sulfolobus solfataricus* and in particular may be a β-glycosidase from *Sulfolobus solfataricus.* Alternatively, the enzyme to be modified may be another member of the glycosyl hydrolase family 1 such as *Pyrococcus furiosus* β-glucosidase, *Dalbergia cochinchinensis* β-glucoside, *Costus speciosus* β-glycoside hydrolase, human lactase phlorizin hydrolase, myrosinase from *Sinapis alba* or *Staphylococcus aureus* phosphogalactosidase.

The amino acid sequence of β-glycosidase from *Sulfolobus solfataricus* is set out in SEQ ID NO:2. Variants in the sequence of SEQ ID NO: 2 may be present in β-glycosidase obtained from other isolates or strains of *Sulfolobus solfataricus* or other cell types expressing β-glycosidases or enzymes classified as being part of the glycosyl hydrolase family 1. Such variants may be modified in accordance with the invention. Carbohydrate processing enzymes, including family 1 glycosyl hydrolases and in particular β-glycosidases from other *Sulfolobus solfataricus* strains or other cell types expressing such enzymes can be isolated following standing cloning techniques, for example, using the polynucleotide sequence of SEQ ID NO: 1 or a fragment thereof as a probe. The isolated enzymes may then be modified.

Preferably, a polypeptide suitable for modification is one which has carbohydrate processing enzymatic activity activity prior to modification, although such activity may be restricted to specific substrates prior to modification. Typically, the modified carbohydrate processing enzyme of the invention will have glycosyl hydrolase, glycosyl synthase and/or transglycosylase activity. The enzyme may possess all three of these activities, any two of them or only one of them. In particular, the enzyme may have glycoside synthase activity or may hydrolyse glycoside substrates. The conditions the enzyme is being used under or the particular concentrations of substrates/products or their ratio may dictate which particular activity an enzyme of the invention displays or which activity predominates at a particular time. In particular, an activated substrate may be used to ensure synthase activity. Alternatively, or additionally, low water activity or sequence modifications may reduce or eliminate hydrolytic activity and allow glycosyl synthase and/or transglycosylase activity to predominate. The conditions and/or concentrations of substrate/products the enzyme of the invention is employed under may be manipulated to ensure that a particular desired activity or activities predominate.

An enzyme in accordance with the present invention is modified such that its activity is modified or increased in comparison to the unmodified form of the enzyme. In particular, the activity of the enzyme is altered to broaden the substrate specificity of the modified enzyme compared to its unmodified counterpart. In particular a modified enzyme of the invention may accept β-mannosides as a

substrate, or other substrates not generally considered to be a natural substrate for the unmodified polypeptide.

The unmodified enzyme may accept a number of different substrates. However, the rate of reaction with different substrates may differ significantly. The unmodified enzyme may have higher affinity for a particular substrate, or subgroup of substrates, within the array of possible substrates that it can act on. The unmodified enzyme will therefore preferentially act on the high affinity substrate(s) even if low affinity substrates are also present at equivalent or higher concentrations. A modification in accordance with the invention may reduce the affinity of the enzyme for one or more of the higher affinity substrates, whilst having no, or little, effect on the affinity of the enzyme for its other substrates. The modifications therefore typically lead to a comparative increase in the activity for other substrates so that the rates of reaction with the variety of different substrates are more closely related and thus the enzyme has in effect a broader substrate specificity. The modified enzyme no longer acts preferentially on particular high affinity substrates but on a wider range of substrates.

The change in substrate specificity may relate to any or all of the activities of the enzyme. For example, it may relate to the hydrolase, synthase and/or transglycosylase activites of the enzyme and in particular to the hydrolase or synthase activities of the enzyme.

The K_M for a particular substrate may be, for example, increased due to the introduction of the modification(s) of the invention by a factor of from 1.1 to 50 fold, preferably by a factor of from 3 to 40 fold, more preferably by a factor of from 5 to 25 fold and even more preferably by a factor of from 10 to 15 fold. This may be accompanied by reduction in K_{CAT} by a factor of from 1.1 to 50 fold, preferably by a factor of from 3 to 40 fold, more preferably by a factor of from 5 to 25 fold and even more preferably by a factor of from 10 to 15 fold for the same substrate. The value of K_{CAT} may be increased, for example, by a factor of from 1.1 to 250, preferably by a factor of from 2 to 200, more preferably by a factor of from 5 to 150, even more preferably by a factor of from 10 to 100 and still more preferably by a factor of from 20 to 75. These changes will typically be seen for a natural substrate of the enzyme and in particular for any of glucoside (Glc), galactoside (Gal), fucoside (Fuc),

xyloside (Xyl) mannoside (Man) and/or glucuronide (GlcA) substrates. In particular, the changes will be seen with glucoside, galactoside, fucoside and/or mannoside substrates and preferably with glucoside and/or galactoside substrates. These changes may occur for any of the modifications of the invention, in particular for a modification at position 432 and/or 433 of SEQ ID No 2 or the equivalent residues. Preferably, these changes will occur for the modifications E432C and/or W433C or for the equivalent substitutions in other glycosyl hydrolases.

The substrate specificity of an enzyme in accordance with the invention can be monitored *in vitro* or *in vivo*, for example in accordance with the methods described in more detail below. In particular, assays can be carried out to monitor activity of the enzyme on particular substrates and in particular glycosidase substrates. Suitable substrates include glucosides, galactosides, fucosides, β-mannosides and β-glucuronides.

The assay may measure glycoside synthesis, hydrolysis and/or transglycosylation. Activity may be assayed using a chromophore such as, for example, paranitrophenol (PNP). The chromophore may be conjugated to a sugar as the carbohydrate donor molecule in glycoside synthesis or transglycosylation or as a substrate for hydrolysis. The release of the chromophore may be monitored to follow the course of the reaction and hence determine the activity of the enzyme. The release of leaving groups such as the fluoride ion, when a glycosyl fluoride is employed as a carbohydrate donor, may also be monitored to determine enzyme activity. The release of the fluoride ions may be measured using a fluoride electrode. Enzyme activity may also be monitored by using mass spectroscopy to monitor the formation of the product ion or decrease in the amount of the substrate ion.

In one aspect, an enzyme according to the present invention incorporates a mutation in at least one of the amino acid residues of 432 (glutamine), 433 (tryptophan) or 439 (methionine) of SEQ ID NO: 2. Alternatively the enzyme of the invention may be a family 1 glycosyl hydrolase comprising at least one mutation at an amino acid residue equivalent to W433, E432 or M439 of SEQ ID NO:2. The invention also encompasses variants of these sequences.

The mutation will typically be an amino acid substitution of W433, E432 or M439 or of the equivalent residues in other family 1 glycosyl hydrolases.

Alternatively, the mutation may be a deletion comprising one or more of these residues or an insertion or duplication affecting these residues. Preferred modifications include mutation of the glutamine, tryptophan or methionine residues or their equivalents to cysteine. Replacement with other amino acids is also contemplated. For example, the residues may be replaced by alanine or valine. In cases where more than one amino acid substitution is made the amino acids introduced may be the same or different at some or all of the sites substituted. For example, the amino acids at positions 432,433 and 439 may all be replaced with cysteine or with any combination of cysteine, alanine and/or valine.

The invention also relates to a variant of SEQ ID NO: 2 having an equivalent modification to those described above. A variant of SEQ ID NO: 2 may be a naturally occurring variant such as one selected from the family 1 of glycosyl hydrolases. A variant may also be a non-naturally occurring variant as described in more detail below. The equivalent amino acid to the residues at positions 432, 433 and 439 of SEQ ID NO: 2 can be identified by aligning a variant peptide with the sequence of SEQ ID NO: 2. The alignment is selected to provide the best possible match to SEQ ID NO: 2. The equivalent amino acid of any such variant to positions 432, 433 or 439 may then be identified and modified. Figure 1 shows an alignment of the amino sequence of several family 1 glycosidases with the three residues equivalent to positions 432, 433 and 439 of SEQ ID No 2 highlighted. By performing similar alignments the equivalent residues can be identified in other family 1 glycosidases and variants and modified. Any of the programs discussed herein may be used to perform the alignment and in particular Clustal W based on BLOSUM 42.

The equivalent amino acid residues to residues 432, 433 and 439 of SEQ ID No 2 will generally be glutamine, tryptophan and methionine respectively. The equivalent amino acids may also be identified by molecular modelling to identify residues playing the equivalent roles to residues 432, 433 and 439 of SEQ ID NO: 2. Typically, such residues will interact with hydroxyl groups of the substrate. A modified polypeptide in accordance with the present invention may comprise one or more of the modifications described herein. Any combination of the modifications described herein may be present.

The carbohydrate processing enzymes of the invention may be further modified to eliminate their hydrolase activity. By replacing the active site catalytic nucleophile of a retaining glycosyl hydrolase it is possible to generate an enzyme which lacks hydrolytic activity, but which is still capable of glycoside synthesis using activated glycosyl donors such as α-glycosyl fluoride. Such mutated enzymes are known as glycosynthases. Existing glycosynthases may be modified in accordance with the invention to give an enzyme with altered substrate specificity. Alternatively, the nucleophilic residue of the active site of a family 1 glycosidase may be mutated at the same time that the other modifications of the invention are introduced.

Any amino acid may be substituted for the nucleophilic amino acid of the active site to generate a glycosynthase. Typically, the nucleophilic amino acid will be replaced by a non-nucleophilic residue. In particular, the nucleophilic residue may be substituted with a glycine, alanine or serine residue and preferably with a serine residue. The mutations Glu387Gly, Glu387Ala, Glu387Ser may be introduced into the sequence of SEQ ID No 2 to generate a glycosynthase or the equivalent mutation may be introduced in other family 1 hydrolases. The equivalent amino acid can be identified by the same means outlined here for identifying the equivalent residues to amino acids 432, 433 and 439 of SEQ ID No 2. Modelling and active site trapping, as well as sequence alignment, may also be used to identify the active site nucleophile which may then be mutated to eliminate the hydrolase activity of the enzyme.

As described above, a variant polypeptide having an amino acid sequence which varies from that of SEQ ID NO: 2 may be modified in accordance with the present invention. A variant for use in accordance with the invention is one having carbohydrate processing enzymatic activity. The variant may be, or be derived from, any family 1 glycosyl hydrolase A modified variant in accordance with the invention is one which preferably demonstrates a broader substrate base compared to a variant sequence not so modified.

In some cases the enzyme may recognise and act on the same substrates as the unmodified enzyme, but to all intents and purposes effectively have a broader substrate range. This is because the modification may make the affinities for various

substrates more equivalent. Prior to modification the enzyme may have particularly high affinity for a small group of substrates out of the possible substrates it can act on. It will therefore preferentially act on that small group of substrates if present. However, post-modification the affinity for those substrates will be reduced and more equivalent to that of other potential substrates. The enzyme will therefore work on a wider range of substrates with equivalent activity.

A variant of SEQ ID NO: 2 may be a naturally occurring variant which is expressed by another strain of *Sulfolobus solfataricus* or other cell type. Such variants may be identified by looking for carbohydrate processing enzymatic activity in those cells which have a sequence which is highly conserved compared to SEQ ID NO: 2. Such proteins may be identified by analysis of the polynucleotide encoding such a protein isolated from an alternative strain, for example, by carrying out the polymerase chain reaction using primers derived from portions of SEQ ID NO: 2 or degenerate primes based on evolutionary conserved regions of SEQ ID NO: 2.

Variants of SEQ ID NO: 2 include sequences which vary from SEQ ID NO: 2 but are not necessarily naturally occurring carbohydrate processing enzymes. Over the entire length of the amino acid sequence of SEQ ID NO: 2, a variant will preferably be at least 30% homologous to that sequence based on amino acid identity. The variant may, for example, be at least 40% homologous, more preferably be at least 50% homologous and still more preferably be more than 65% homologous to the amino acid sequence of SEQ ID NO: 2. In some embodiments the polypeptide will be at least 75% homologous, preferably at least 80% homologous and even more preferably the polypeptide is at least 85% homologous to SEQ ID NO: 2. The polypeptide may be at least 90% homologous and still more preferably be at least 95%, 97% or 99% homologous to the amino acid sequence of SEQ ID NO: 2. A variant may be a variant of any family 1 glycosyl hydrolase with one of the percentages of sequence homology specified above. In particular, a variant may be a variant of any of those proteins shown in Figure 1 with any of the percentages of sequence homology specified herein to that sequence.

These percentages of homology may, for example, be over at least 30 amino acids, preferably over at least 40 amino acids and even more preferably over 50 amino acids. The percentages of homology may be over at least 75 amino acids,

preferably at least 100, more preferably over 150 amino acids and in some cases will be over the entire length of the variant. In some cases they may be over all but 10, preferably all but 20, more preferably all but 30 and even more preferably all but 50 contiguous amino acids of the variant. There may be at least 80%, for example at least 85%, 90% or 95%, amino acid identity over a stretch of 40 or more, for example 60, 100 or 120 or more, contiguous amino acids ("hard homology").

In a preferred embodiment of the invention the variant will comprise a region which has one of the levels of amino acid sequence homology specified herein to amino acids 425 to 450 of SEQ ID No.2. Alternatively, the variant may comprise a region which has such a degree of sequence homology to the equivalent region to amino acids 425 to 450 of SEQ ID No. 2 from a different family 1 glycosyl hydrolase and in particular to one of such regions as depicted in Figure 1.

Preferably sequence alignment and the determination of homology may be performed using ClustalW based on a BLOSUM42 matrix.

The variant may be one with any of the values of percentage homology mentioned herein to any of the proteins listed in Figure 1 (either to the entire protein sequence of the protein or to the partial sequences shown in Figure 1). The variant may be one of any family 1 hydrolase as long as one or more of the residues equivalent to 432, 433 or 439 has been modified.

Amino acid substitutions may be made to the amino acid sequence of SEQ ID NO: 2, for example from 1, 2 or 3 to 10, 20 or 30 substitutions. Such modifications may be introduced into any family 1 glycosyl hydrolase. Conservative substitutions may be made, for example, according to the following table. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar – uncharged	S T M
		N Q
	Polar – charged	D E
		K R
AROMATIC		H F W Y

One or more amino acid residues of the amino acid sequence of SEQ ID NO: 2 may alternatively or additionally be deleted. From 1, 2 or 3 to 10, 20 or 30 residues may be deleted, or more. Polypeptides of the invention also include fragments (c) of the above-mentioned sequences. Such fragments retain carbohydrate processing enzymatic activity. Fragments may be at least from 10, 12, 15 or 20 to 60, preferably100 or 200, 300 or more amino acids in length.

Such fragments may be used to produce chimeric enzymes using portions of enzyme derived from other carbohydrate processing enzymes such as, for example, glycosidases.

One or more amino acids may be alternatively or additionally added to the polypeptides described above. An extension may be provided at the N-terminus or C-terminus of the amino acid sequence of SEQ ID NO: 2 or polypeptide variant or fragment thereof. The, or each, extension may be quite short, for example from 1 to 10 amino acids in length. Alternatively, the extension may be longer. A carrier protein may be fused to an amino acid sequence according to the invention. A fusion protein incorporating the polypeptides described above can thus be provided.

Polypeptides of the invention may be in a substantially isolated form. It will be understood that the polypeptide may be mixed with carriers or diluents which will not interfere with the intended purpose of the polypeptide and still be regarded as substantially isolated. A polypeptide of the invention may also be in a substantially purified form, in which case it will generally comprise the polypeptide in a preparation in which more than 90%, e.g. 95%, 98% or 99%, by weight of the polypeptide in the preparation is a polypeptide of the invention.

Polypeptides of the invention may be modified for example by the addition of histidine residues to assist their identification or purification or by the addition of a signal sequence to promote their secretion from a cell where the polypeptide does not naturally contain such a sequence. It may be desirable to provide the polypeptides in a form suitable for attachment to a solid support. For example the polypeptides of the invention may be modified by the addition of a cysteine residue.

A polypeptide of the invention above may be labelled with a revealing label. The revealing label may be any suitable label which allows the polypeptide to be detected. Suitable labels include radioisotopes, e.g. ^{125}I, ^{35}S, enzymes, antibodies,

polynucleotides and linkers such as biotin. Labelled polypeptides of the invention may be used in diagnostic procedures such as immunoassays in order to determine the amount of a polypeptide of the invention in a sample.

The proteins and peptides of the invention may be made synthetically or by recombinant means. The amino acid sequence of proteins and polypeptides of the invention may be modified to include non-naturally occurring amino acids or to increase the stability of the compound. When the proteins or peptides are produced by synthetic means, such amino acids may be introduced during production. The proteins or peptides may also be modified following either synthetic or recombinant production.

The proteins or peptides of the invention may also be produced using D-amino acids. In such cases the amino acids will be linked in reverse sequence in the C to N orientation. This is conventional in the art for producing such proteins or peptides.

A number of side chain modifications are known in the art and may be made to the side chains of the proteins or peptides of the present invention. Such modifications include, for example, modifications of amino acids by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$, amidination with methylacetimidate or acylation with acetic anhydride.

The polypeptides of the invention may be introduced into a cell by *in situ* expression of the polypeptide from a recombinant expression vector. The vector may be stably integrated into the genome of the cell. The expression vector optionally carries an inducible promoter to control the expression of the polypeptide.

Such cell culture systems in which polypeptides of the invention are expressed may be used in assay systems.

A polypeptide of the invention can be produced in large scale following purification by high pressure liquid chromatography (HPLC) or other techniques after recombinant expression as described below.

The enzymes of the present invention are modified. By this it is meant that one or more amino acid sequence changes have been introduced into the enzyme in comparison to the unmodified sequence of the protein. Thus, typically a wild type enzyme will have had amino acid sequence changes introduced to produce the

modified enzyme. The amino acid sequence changes introduced will affect amino acid positions 432, 433 and/or 439 of SEQ ID NO: 2 or the equivalent residues of other family 1 glycosyl hydrolases. The unmodified form of the enzyme will typically be the naturally occurring form of the enzyme. However, the amino acid substitutions of the invention may also be introduced into mutant and variant forms of family 1 glycosyl hydrolases.

In a preferred embodiment of the invention the enzyme is a modified form of β-galactosidase of *Sulfolobus solfataricus*, β-galactosidase of *Sulfolobus shibatae*, β-galactosidase of *Sulfolobus acidocaldarius*, β-galactosidase of *Thermoplasma volcanium*, β-galactosidase of *Pyrococcus furiosus*, β-glycosidase of *Agrobacterium tumefaciens*, β-D-glucoside glucohydrolase of *Bacillus circulans*, β-D-glucoside glucohydrolase of *Agrobacterium sp.*, β-glucoside of *Rhizobium meliloti*, β-D-glucoside of *Bacillus halodurans*, β-D-glucoside glucohydrolase of *Paenibacillus polymyxa*, β-galactosidase glucohydrolase of *Pyrococcus woesi*, β-glucoside of *Dalbergia cochinchinensis*, Furostanol β- glucoside of *Costus specious*, Lactase phlorizin hydrolase of *Homo sapiens*, Myrosinase of *Sinapis alba*, or 6-phospho-beta-galactosidase of *Staphylcoccus aureus* which comprises one or more of the modifications of the invention. The -1 binding pocket for each of these enzymes is depicted in Figure 1. The sequences are aligned to residues 425 to 450 of the β-galactosidase of *Sulfolobus solfataricus*. A modified polypeptide of the invention may comprise any of the sequences depicted in Figure 1 into which one or more of the modifications of the invention have been introduced. A modified polypeptide of the invention may comprise a variant of such sequences.

The invention also relates to polynucleotides encoding the modified carbohydrate processing enzymes. A polynucleotide of the invention typically is a contiguous sequence of nucleotides which is capable of hybridising selectively with the coding sequence of SEQ ID NO: 1 or to the sequence complementary to that coding sequence. Polynucleotides of the invention include variants of the coding sequence of SEQ ID NO: 1 which encode the amino acid sequence of SEQ ID NO: 2. Such polynucleotides additionally incorporate one or more modification to encode a modified polypeptide as described in more detail above.

A polynucleotide for use in the invention and the coding sequence of SEQ ID NO: 1 can typically hybridize at a level significantly above background or alternatively the complement of such a sequence can. Background hybridization may occur, for example, because of other cDNAs present in a cDNA library. The signal level generated by the interaction between a polynucleotide of the invention and the coding sequence of SEQ ID NO: 1 is typically at least 10 fold, preferably at least 100 fold, as intense as interactions between other polynucleotides and the coding sequence of SEQ ID NO: 1. The intensity of interaction may be measured, for example, by radiolabelling the probe, e.g. with ^{32}P. Selective hybridization is typically achieved using conditions of medium to high stringency (for example 0.03M sodium chloride and 0.003M sodium citrate at from about 50°C to about 60°C).

A nucleotide sequence capable of selectively hybridizing to the DNA coding sequence of SEQ ID NO: 1 or to the sequence complementary to that coding sequence will be generally be at least 30%, preferably at least 40% and even more preferably at least 50% homology to the coding sequence of SEQ ID No. 1. Sequence homology corresponds to sequence identity. In some embodiments it will be at least 60%, preferably at least 70% and more preferably at least 80%, homologous to the coding sequence of SEQ ID NO: 1 or its complement over a region of at least 20, preferably at least 30, for instance at least 40, 60 or 100 or more contiguous nucleotides or, indeed, over the full length of the coding sequence. Thus there may be at least 85%, at least 90% or at least 95% nucleotide identity over such regions.

Any combination of the above mentioned degrees of homology and minimum size may be used to define polynucleotides of the invention, with the more stringent combinations (i.e. higher homology over longer lengths) being preferred. Thus for example a polynucleotide which is at least 85% homologous over 25, preferably over 30, nucleotides forms one aspect of the invention, as does a polynucleotide which is at least 90% homologous over 40 nucleotides.

Nucleotide homology may be determined using various BLAST programs and in particular PSI-BLAST. Polynucleotide variants for use in the invention may be identified by performing PSI-BLAST searches of SWISSPROT and TREMBL to

a family 1 glycosyl hydrolase, including any of those mentioned herein, and in particular to the amino acid sequence of SEQ ID No. 1.

Alternatively, the UWGCG Package provides the BESTFIT program which can be used to calculate homology (for example used on its default settings) (Devereux *et al* (1984) *Nucleic Acids Research* 12, p387-395). The PILEUP and BLAST algorithms can be used to calculate homology or line up sequences (such as identifying equivalent or corresponding sequences (typically on their default settings), for example as described in Altschul S. F. (1993) J Mol Evol 36:290-300; Altschul, S, F *et al* (1990) J Mol Biol 215:403-10.

Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pair (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighbourhood word score threshold (Altschul *et al*, supra). These initial neighbourhood word hits act as seeds for initiating searches to find HSP's containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extensions for the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1992) *Proc. Natl. Acad. Sci.* USA 89: 10915-10919) alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

The BLAST algorithm performs a statistical analysis of the similarity between two sequences; see e.g., Karlin and Altschul (1993) *Proc. Natl. Acad. Sci.* USA 90: 5873-5787. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by

chance. For example, a sequence is considered similar to another sequence if the smallest sum probability in comparison of the first sequence to the second sequence is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

Polynucleotides of the invention may comprise DNA or RNA. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to polynucleotides are known in the art. These include methylphosphate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art. The invention also includes protein nucleic acid (PNA) molecules comprising the sequences of the invention.

Polynucleotides of the invention may be used to produce a primer, e.g a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40 nucleotides in length, and are also encompassed by the term polynucleotides of the invention as used herein. The invention also provides a microarray comprising such polynucleotides.

Polynucleotides such as a DNA polynucleotide and primers according to the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques. The polynucleotides are typically provided in isolated and/or purified form.

In general, primers will be produced by synthetic means, involving a step wise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

Longer polynucleotides will generally be produced using recombinant means, for example using PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of about 15-30 nucleotides) to a region of the

gene which it is desired to clone, bringing the primers into contact with DNA obtained from a suitable cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

Although in general the techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook *et al*, 1989.

Polynucleotides or primers of the invention may carry a revealing label. Suitable labels include radioisotopes such as ^{32}P or ^{35}S, enzyme labels, or other protein labels such as biotin. Such labels may be added to polynucleotides or primers of the invention and may be detected using techniques known *per se*.

Polynucleotides of the invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells are described below in connection with expression vectors.

Preferably, a polynucleotide of the invention in a vector is operably linked to a control sequence which is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. Such expression vectors can be used to express the polypeptide of the invention.

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences. Multiple copies of the same or different modified carbohydrate processing enzyme genes may be introduced into the vector.

Such vectors may be transformed into a suitable host cell to provide for expression of a polypeptide of the invention. Thus, a polypeptide according to the invention can be obtained by cultivating a host cell transformed or transfected with an expression vector as described above under conditions to provide for expression of the polypeptide, and recovering the expressed polypeptide.

The vectors may be for example, plasmid, virus or phage vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vector may be an artificial chromosome such as a human or yeast artificial chromosome. The vectors may contain one or more selectable marker genes, for example a tetracycline resistance gene. Promoters and other expression regulation signals may be selected to be compatible with the host cell for which the expression vector is designed. Multiple copies of the same or different modified glycosidase gene in a single expression vector, or more than one expression vector each including a modified glycosidase gene which may be the same or different may be transformed into the host cell.

Host cells transformed (or transfected) with the polynucleotides or vectors for the replication and expression of polynucleotides of the invention will be chosen to be compatible with the said vector. In one embodiment of the invention lypholised host cells are produced and used directly as biocatalysts.

The present invention also provides non-human animals comprising a polynucleotide encoding a modified enzyme of the invention. The non-human transgenic animal may, for example, be a rodent, such as a mouse or rat, or an animal such as a pig, sheep or cow. The invention also provides a plant comprising a polynucleotide encoding a modified polypeptide of the invention.

Where the amino acid at position 433, 432 or 439 is substituted by cysteine, the cysteine may be chemically modified so as to change the substrate specificity of the enzyme. The cysteine may be modified so as to comprise a positively-charged group, a negatively-charged group or an uncharged group. The positively charged group may be of formula $-(CH_2)n-N^+R_3$, wherein n is a positive integer from 1 to 4 and each R, which may be the same or different, is H or a C_1-C_4 alkyl group (preferably a methyl group). A preferred positively charged group is

$-CH_2CH_2NMe_3^+$. The negatively-charged group may be of formula $-(CH_2)n-SO_3^-$ or $-(CH_2)n-COO^-$, wherein n is a positive integer from 1 to 4. Preferably, the negatively-charged group is $-CH_2CH_2-SO_3^-$. The uncharged group may be a C_1-C_4 alkyl group and preferably is methyl.

An enzyme in accordance with the invention can be used *in vitro*, for example, bound to an immobile substrate. The enzyme can be immobilised through the addition of a binding sequence such as a His-tag or maltose binding site or by using a general immobiliser. The immobilised enzyme can then be used in the ring expansions and conversions described above.

The activity of a modified enzyme in accordance with the invention may be monitored by carrying out assays *in vitro* or *in vivo*, that is within a host cell, to monitor for carbohydrate processing activity of the enzyme. Such assays may include monitoring for the production of glycosides.

The modified enzymes in accordance with the present invention can be used in any methods involving glycosyl synthase, transglycosylase and/or hydrolase activity using glycoside substrates. They can be used wherever it is desired to a form β glycoside bond. In a particularly preferred aspect of the present invention, the enzymes are used in methods in which one or more glycoside substrates, such as one or more glucoside, galactoside, fucoside, mannoside or glucuronide substrates are incubated together with the modified enzyme. Preferably, the glycoside is β-mannoside. Preferably, in accordance with present invention more than one substrate is provided in the same reaction vessel to yield a library of different glycosides. Such substrates may include a natural substrate of the unmodified polypeptide and one or more non-natural substrates, that is substrates that are not usually accepted by the unmodified polypeptide. Thus methods may take advantage of the broadened substrate specificity of the enzymes of the invention to produce a variety of products in a single reaction vessel. Alternatively, reactions may be run in parallel using the enzyme of the invention where the only change between reactions is that a different substrate is employed and hence a different glycoside produced. Such reactions may be run in multiwell plates to allow for the individual screening of each glycoside produced in a high throughput assay.

The enzymes of the invention may be used in glycoside synthesis and in transglycosylation, they may also be employed in glycoside hydrolysis. Using the enzymes practically any β glycoside linkage may be synthesised or alternatively hydrolysed. In embodiments of the invention where the aim is glycoside synthesis the enzyme may be modified so that it is a glycosynthase i.e. the active site nucleophile will have been eliminated and replaced with an alternative amino acid. In such cases, typically the carbohydrate donor will be an activated donor such as a fluoryl or PNP linked carbohydrate donor. The enzyme catalyses the transfer of the glycoside, onto a chosen alcohol acceptor such as, for example another saccharide or polypeptide. In a preferred example, the glycosyl donor used is be a β-D-mannoside and it is used to form Man β(1,4) Glc NAc.

The enzymes of the invention may be used to generate an array of molecules conjugated to carbohydrates. They may be used to generate glycoproteins and in particular O-linked glycosylations, where typically the sugar group is conjugated to a serine or a threonine residue. The enzymes may be used to help produce recombinant proteins which have the same or similar glycosylations to naturally occurring versions of the proteins. The enzymes may be used to generate antibiotics and in particular macrolide antibiotics. They may be used in the food industry, for example to achieve depulping. They may also be used in detergents.

The enzymes may be used in therapy both as therapeutic molecules themselves and in the generation of therapeutic molecules. Thus the enzymes may be used in the treatment of a human or animal subject. The enzymes may be used in methods of treatment of the human or animal body by surgery or therapy.

The enzymes may be used to develop glycoconjugates for use in LEAP (lectin enzyme activated prodrug system). Lectins are found on the surface of cells. There are a variety of different lectins with certain ones only being found on a specific cell type or on specific groups of cell types. In LEAP glycoconjugates comprising a carbohydrate group capable of binding a specific lectin and an enzyme capable of activating a prodrug are generated and administered to a subject to which the prodrug is also given. The lectin binding group of the conjugate targets it to the specific cell type or types expressing the target lectin and hence the prodrug is only activated at the surface of the specific cell types. Thus LEAP allows drugs to be

targeted to a specific class of cells through the lectins that they express and this can be used for a variety of functions including eliminating undesired cells. LEAP is described in WO 02/080980 which is incorporated herein by reference in its entirety. The enzymes of the invention can be employed in the production of any of the glycoconjugates described in WO 02/080980.

In glycoside synthesis using the enzyme of the invention the molecule glycosylated may be a saccharide or a different molecule such as a polypeptide. Multiple glycosylations of the same molecule may occur and, for example, di-, tri-, tetra or oligosaccharides may be generated. These may be generated, for example, by multiple step-wise glycosyl additions or by addition of an oligosaccharide to the target molecule. Branched oliosaccharides may also be added to a target molecule using the enzyme of the invention.

Example 1

The binding domain of the thermophilic, retaining, exo-β-glycosidase, from *Sulfolobus solfataricus* (SSβG, EC 3.2.1.23) was probed using site directed mutagenesis. The gene encoding this enzyme, was originally isolated and sequenced from the *Sulfolobus solfataricus* strain MT4 (Cubellis *et al., Gene* (1990) 94, 89-94) and is classified as a member of the glycosyl hydrolase family 1 (Henrissat, (1991) *Biochem J.*, 280, 309-316). This robust, thermophilic enzyme is ideal (Pisani *et al., Eur. J. Biochem.* **187** (1990) 321-328; Moracci *et al., Protein Eng.*, **9** (1996) 1191-1195; and Nucci *et al., Biotechnol. Appl. Biochem.*, **17** (1993) 239-250). It can be routinely expressed in *Escherichia coli* (Moracci *et al., Enzym. Microb. Technol.*, **17** (1995) 992-997). Its 3D structure has a classic $(\alpha/\beta)_8$ TIM barrel (Banner *et al., Nature* **255** (1975) 609-614) containing a radial active site channel in a kink of the 5th α/β repeat (Aguilar *et al., J. Mol. Biol.*, **271** (1997) 789-802). Substrate specificity in this enzyme is associated with two residues in the binding site, glutamate 432 and methionine 439 which are largely conserved across family 1 glycosyl hydrolases (Figure 1). Importantly, those family 1 hydrolases in which these residues differ also show altered substrate specificities (*vide infra*). In the examples below we have analyzed the structure of SSβG and created point mutants in which key residues implicated in specificity determination have been tailored. This results

in robust mutant enzymes with altered substrate specificities and enhanced synthetic utility.

Materials and methods

Reagents, enzymes and bacterial strains

The wild type sequence, *lac S*, encoding the β-glycosidase from *Sulfolobus solfataricus* (SsβG), was amplified by PCR from *Sulfolobus* genomic DNA, using the following primers:

5': CCATGGGACACCACCACCACCACCACTCATTAC (SEQ ID No.19)

3': CTCGAGTTAGTGCCTTTATGGCTTTACTGGAGGTAC (SEQ ID No.20)

The 5' primer introduced an N-terminal *Nco* I site and a 6 x His tag immediately following the ATG initiation codon. The 3' primer introduced a *Xho* I site after the stop codon. The PCR product was cloned into pCR2.1 (Invitrogen) and individual clones were sequenced to verify that no errors had been introduced.

Electrocompetent *Escherichia coli* strain BL21(DE3) and His-bind Nickel resin were obtained from Novagen. 4-Methylumbelliferyl-β-D-glycoside substrates were purchased from Sigma. *Pfu*-turbo DNA polymerase was obtained from Stratagene and *Nco* I, *Xho* I restriction endonucleases, T4 DNA ligase from Promega, UK. Oligonucletoide primers were obtained from MWG BioTech GmBH and Cruachem Ltd. DNA sequencing was carried out by the DNA Sequencing Service, Dept. Biological Sciences, Durham, using standard protocols on Applied Biosystems DNA Sequencers.

Construction, selection and screening of the single point mutants

Mutations were introduced into the *lac S* gene coding sequence (in pCR2.1) according to the Stratagene QuickChange mutagenesis system, using the suppliers' protocols. Oligonucleotide primers used for the generation of the point mutations were:

for Glu-432→Cys;

5'TCTAGCTGATAATTACTGTTGGGCTTCAGGATTCT-3' (SEQ ID NO: 21);

for Trp-433→Cys;

5'-CTAGCTGATAATTACGAATGTGCTTCAGGAT TCTC-3 (SEQ ID NO: 22);

for Met-439→Cys;

5'-GCTTCAGGATTCTCTTGTAGGTTTGGTCTG-3'(SEQ ID NO: 23)

along with the corresponding complementary primers. Individual point mutations were verified by DNA sequence analysis. Wild type and mutated coding sequences were cloned into the *Nco* I / *Xho* I sites of expression vector pET-24-d(+) (Novagen) and transformed into *E. coli* BL21(DE3). Putative transformants were identified by colony PCR using the SSβG coding sequence primers. Selected clones were checked by DNA sequencing to confirm the mutation, and the absence of unintended PCR-introduced base changes.

Overexpression and purification of the His_6-tagged mutant enzymes

Selected clones were grown in LB medium containing kanamycin (50 μg/ml), at 37°C to an O.D. of 0.6 at 600 nm, and the target were proteins induced by the addition of 0.1M IPTG. Cells were harvested by centrifugation, resuspended in 1/10th volume of column loading buffer (5mM imidazole, 20mM Tris, 0.5M NaCl, pH 7.8), and lysed using a Soniprep 150 Sonicator. The suspension was recentrifuged to pellet cell debris (10000 rpm, 30 min), and the His_6-tagged recombinant proteins were purified from the supernatant using Ni-chelation chromatography (wash buffer, 60mM imidazole, 20mM Tris, 0.5M NaCl, pH 7.8; elution buffer 300mM imidazole, 20mM Tris, 0.5M NaCl, pH 7.8). The eluted protein peak was dialysed against 50mM sodium phosphate buffer, (pH 6.5), and stored at 4°C. Protein concentration was quantified by the method of Bradford 1976 *Anar Biochem.*, 151, 196-204 (reagents from Biorad, Netherlands). Purified proteins were analysed by SDS-polyacrylamide gel electrophoresis, gel fitration chromatography and ESMS (Micromass LCT, ± 8Da).

Characterisation of the kinetic properties of enzymes

Parameters were determined by the method of initial rates. Activity was measured in time course assays of the hydrolysis of 4-methylumbelliferyl-β-D-glycosides (β-D-gluco, β-D-galacto, β-D-fuco, β-D-manno, β-D-xylo, β-D-glucurono) at 5-15 concentrations (0.001-1.5 mM) incubated at 80°C in 50 mM sodium phosphate buffer, pH 6.5. Reactions were terminated at 2, 5, 10, 15 min by

the addition of 100µl of ice cold 1M Na_2CO_3, pH 10 and analyzed (Labsystems Fluoroscan Ascent plate reader, excitation 460 nm, emission 355 nm). K_M and k_{cat} were derived by fitting the initial rates to hyperbolic Michaelis-Menten curves using GraFit 4 (Erithacus Software Ltd, Staines, UK).

Sequence Analysis

Sequence alignment was performed using ClustalW based on a BLOSUM42 matrix. Enzymes of interest were determined by their sequence similarity using PSI-BLAST searches of SWISSPROT and TREMBL to BGAL_SULSO (SSβG), *Sulfolobus solfataricus* β-glycosidase (SSβG) (Cubellis *et al., supra*) including, *Pyrococcus furiosus* β-glucosidase (*CelB*) (PFβG) (Voorhurst *et al., J. Bacteriol* (1995) 177, 7105-7111) used for molecular mechanics analysis. In this way several glycosidases were also identified with both altered substrate specificity and differences in the residues occupying positions 432, 433 and 439 (SSβG numbering): *Dalbergia cochinchinensis* Dalcochinin-8'-*O*-β-glucoside β-glucosidase (Cairns *et al., supra*, TREMBL accession No. Q95PK3); *Costus speciosus* furostanol-26-*O*-β-glycoside hydrolase (Inoue et al FEBS letters 1996 389, 273-277; LPH_HUMAN, human lactase phlorizin hydrolase (Mantei *et al.*, Supra); MY3_SINAL, myrosinase from *Sinapis alba* (Xue *et al.,supra*); LACG_STAAU (6-PBG), *S. aureus* 6-phosphogalactosidase (Breidt and Steward, *supra*).

Molecular Mechanics and Docking Analysis

The X-ray structure of SSβG (RCSB-PDB entry 1gow) was used as the starting point for calculations. The enzyme setup was performed with Insight II, version 2.3.0 (Accelerys Inc. San Diego, CA, USA). To create initial coordinates for the minimization, hydrogens were added at the pH used for kinetic measurements (6.5). The model system was solvated with a 5 Å layer of water molecules. Energy simulations were performed with the DISCOVER module within Cerius2, Version 3.8 on a Silicon Graphics Indigo computer, using the consistent valence force field (CVFF) function. A non-bonded cutoff distance of 18 Å with a switching distance of 2 Å was employed. The non-bonded pair list was updated every 20 cycles and a dielectric constant of 1 was used in all calculations. Docked structures were

generated using the Builder module, and aligned within the active site using appropriate bump, hydrogen bonding and docking interaction monitors. The enzyme was then minimized in stages, with initially only the water molecules being allowed to move, followed by water molecules and the amino acid side chains, and then finally the entire enzyme. The β-D-Glc*p* was free to move throughout all stages of the minimization. Each stage of energy minimization was conducted by means of the method of steepest descents without Morse or cross terms until the derivative of energy with respect to structural perturbation was less than 5.0 kcal/Å; then the method of conjugate gradients, without Morse or cross terms until the derivative of energy with respect to structural perturbation was less than 1.0 kcal/Å; and finally the method of conjugate gradients, *with* Morse and cross terms until the final derivative of energy with respect to structural perturbation was less than 0.1 kcal/Å.

Glycoside synthesis

Enzyme (WT, W443C or E432C, 1mg) was added to a mixed solution (1 mL) of *para*-nitrophenyl (pNP) β-D-manno-, galacto-, gluco- and xylo- pyranosides (0.03 mmol of each) in 1:9 MeOH:phosphate buffer (pH 6.5) and incubated at 50 °C for 45 min (WT), 4h (WT), 8h (W433C, E432C). After this time the solutions were extracted with EtOAc to remove *para*-nitrophenol and passed through short Sephadex and Celite:Graphite (1:1) columns to remove protein, pNP-glycoside and remaining *para*-nitrophenol. Solvent was removed and product mixtures were analysed by ^{1}H NMR and ESMS. Yields based on donor were calculated from integration of anomeric proton resonances in ^{1}H NMR (D_2O, 500MHz): α-Gal (δ 5.12, d, J 4.0Hz), α-Glc (δ 5.08, d, 3.8 Hz), α-Xyl (δ 5.04, d, J3.4 Hz), α-Man (δ 5.03, d, J 1.8Hz), β-Man (δ 4.75, s), β-Glc (δ 4.49, d, J8.0Hz), β-Gal (δ 4.45, d, 7.9Hz), Me-β-Man (δ 4.44, s), β-Xyl (δ 4.42, d, J7.8Hz), Me-β-Glc (δ 4.23, d, J 8.1Hz), Me-β-Xyl (δ 4.18, d, J 7.8Hz), Me-β-Gal (δ 4.17, d, J 8.0Hz).

Results

Analysis of the binding site of SSβG

In an attempt to dissect the specificity determining interactions of SSβG with its substrates we examined the 3D structures of SSβG (RCSB–PDB 1gow) and the close structural homologue *B. polymyxa* β-glycosidase (BPβG). Valuably, 3D structures of BPβG containing D-gluconate bound as a substrate mimic (1bgg) and a 2-deoxy-2-fluoro-α-D-glucosyl-enzyme intermediate have recently been reported. This allowed homology modelling and docking analysis of SSβG to create a minimum energy structure through molecular mechanics containing β-D-glucopyranose as a substrate mimic. Both the structures of BPβG and SSβG showed that the conserved residues E432 and W433 (SSβG numbering) (Figure 1) create vital hydrogen bonds to the OH-4 and 3, respectively, of their substrates. Furthermore, M439 sits at the base of the small side pocket that lies in close proximity to OH-6. Gratifyingly, sequence analysis (Figure 1) supports the identification of the potential of these residues in specificity determination: e.g., S432 (SSβG numbering) rather than E432 in the phosphogalactosidase (E.C. 3.2.1.85) from *S. aureus* (Breidt and Stewart, *supra*), and G433 rather than W433 in the broad specificity glycosidase/cerebrosidase human lactase phlorizin hydrolase (E.C. 3.2.1.62) (Mantei *et al., supra*).

We therefore selected E432, W433 and M439 for mutagenesis as potentially critical active site residues for determining substrate specificity. Cysteine was chosen as the target residue for mutations, as a single flexible residue that could play a variety of roles. C behaves in proteins similarly to W and M, is structurally close to S but would alter some of the key interactions identified (e.g., abolish hydrogen bonding) in a conservative, informative manner.

Construction and kinetic characterisation of WT and mutant enzymes

SSβG-WT, -E432C, -W433C and –M439C enzymes were expressed in *E. coli* as recombinant proteins containing an N-terminal His_6-tag to avoid interfering with the critical multimer-forming interactions of the C-terminus of the protein. Yields of recombinant protein were of the order of 15 mg per litre of culture. The purified, recombinant WT and mutated SSβG proteins gave single bands on SDS-PAGE at an indicated approx mol. wt. of 57,000, and gave a single peak on analysis by gel filtration under non-denaturing conditions, of an indicated molecular weight

consistent with the formation of dimeric molecules (data not presented). Exact masses were confirmed by ESMS (± 8 Da). Both WT and mutant recombinant SSβGs were >95% pure by these analyses.

Determination of the Michaelis-Menten parameters for the WT and mutant enzymes was performed at pH 6.5 at 80° C for a broad range of representative, fluorophore-containing 4-methylumbelliferyl glycoside substrates, which allowed activities to be determined with a high degree of sensitivity (Table). Under these optimized assay conditions, the glucoside (Glc), galactoside (Gal) and fucoside (Fuc) substrates were hydrolysed well by SSβG-WT, but the xyloside (Xyl) substrate was hydrolysed relatively poorly (approx. 3% of turnover as determined by k_{cat} compared with β-D-glucoside). Interestingly, low levels of previously undetected β-D-mannoside (Man) and β-D-glucuronide (GlcA) activities (approx. 1% and 0.5% of turnover towards β-D-glucoside) were observed. In all cases the absolute D-stereospecificity and β-stereoselectivity of SSβG was maintained and no activity was detected towards L- or α-glycoside substrates.

Substrate	Enzyme, SSβG-	K_m, mM	k_{cat}, s^{-1}	k_{cat} / K_m, $s^{-1}mM^{-1}$
4-MUGlc	WT	0.046 ± 0.017	140 ± 20	2900
	E432C	0.34 ± 0.07	5.1 ± 0.5	15
	W433C	1.61 ± 0.35	33 ± 5	20
	M439C	0.068 ± 0.028	190 ± 40	2900
4-MUGal	WT	0.066 ± 0.017	98 ± 7	1490
	E432C	0.47 ± 0.14	5.4 ± 0.8	11
	W433C	2.2 ± 1.2	14 ± 6	6.3
	M439C	0.083 ± 0.016	94 ± 11	1130
4-MUFuc	WT	0.011 ± 0.002	80 ± 2	7300
	E432C	0.34 ± 0.04	18 ± 1	53
	W433C	0.41 ± 0.09	31 ± 3	76
	M439C	0.023 ± 0.005	91 ± 8	4000
4-MUMan	WT	0.036 ± 0.009	1.8 ± 0.2	50
	E432C	0.90 ± 0.26	2.8 ± 0.7	3.2
	W433C	0.18 ± 0.02	0.92 ± 0.05	5.1
	M439C	0.042 ± 0.015	2.3 ± 0.4	53
4-MUXyl	WT	0.13 ± 0.03	3.8 ± 0.3	30
	E432C	1.26 ± 0.21	2.8 ± 0.3	2.2
	W433C	0.59 ± 0.19	1.5 ± 0.3	2.5
	M439C	0.068 ± 0.007	9.3 ± 0.2	136
4-MUGlcA	WT	1.3 ± 0.4	0.81 ± 0.18	0.60
	E432C	NAD[a]	NAD	NAD
	W433C	NAD	NAD	NAD
	M439C	1.4 ± 0.6	1.3 ± 0.4	0.92

It is apparent that the E432C and W433C mutations have a dramatic effect upon activity towards certain substrates. Glc k_{cat}/K_M is reduced 200-fold and 140-fold, and Gal k_{cat}/K_M is reduced 130-fold and 230-fold for E432C and W433C respectively. However, although Man, Xyl activities were also reduced, these reductions were far less marked to k_{cat}/K_M values only 10-16-fold lower than WT for E432C and W433C. Consistent with the prediction that hydrogen bonds to OH-4 (E432C) and OH-3 (W433C) are abolished in these mutants, these k_{cat}/K_M decreases correspond to a loss of affinity of approx. 4.5-10.5 $kJ.mol^{-1}$. These reductions in k_{cat}/K_M were largely manifested in reductions in ground state binding with K_M values generally increased up to 37-fold; the greatest K_M increases in both W433C and E432C were observed for Glc, Gal, Fuc. Variations in k_{cat} in the mutants E432C and W433C were less uniform; there were large overall reductions in Gal,Glc turnover (k_{cat} decreased by approx. 5- to 30-fold), whereas k_{cat} for Fuc, Xyl, Man in E432C and W433C are essentially similar to those for SSβG-WT (2-fold *increased* k_{cat} (Man) for E432C to only 2.9-fold lowered k_{cat} (Xyl) for W433C). This indicates that an additional transition state destabilisation is induced by mutation in E432C and W433C that essentially affects Gal,Glc only.

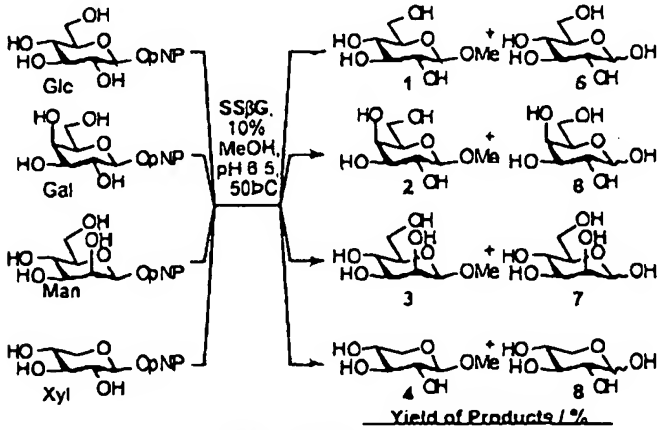
We were pleased to discover that as a result of the varying alterations in k_{cat}/K_M for different substrates the specificities of E432C and W433C were remarkably more broad than SSβG-WT. For example, the variation of k_{cat}/K_M for Glc:Gal:Xyl:Man moves from a restrictive 100-fold specificity range for WT to a broad 8-fold range for W433C (WT, 100:52:1:2 → W433C, 8.1:2.5:1:2).

The M439C mutation has a more subtle effect on specificity than the E432C and W433C mutations. Consistent with the ability of M439 to modulate substrate C-6 substituent specificity suggested by molecular modelling, the level of k_{cat}/K_M alteration caused by mutation differs according to C-6 structure. M439C shows almost identical values to WT for Gal, Glc, Man substrates in which the CH_2OH at C-6 is unaltered. However, k_{cat}/K_M for Fuc, which instead bears a CH_3 at C-6, is 1.8-fold lower than WT and excitingly, k_{cat}/K_M for Xyl, which bears no C-6 substituent, is 4.7-fold higher than WT. It should also be noted that, the mutation has the effect of increasing k_{cat} for all the substrates, suggesting that a general stabilisation of the transition states is occurring.

It has been proposed previously that in other family 1 glycosidases the position corresponding to E432 in SSβG is responsible for the modulation of carbohydrate substrate *O*-6 substituent binding and in particular the rejection of negatively charged substituents (Aguilar *et al., supra*). Contrary to this prediction, the E432C mutant has no detectable activity towards GlcA, which at pH 6.5 bears a negative charge at C-6. In contrast, M439C shows slightly enhanced k_{cat}/K_M values for GlcA (1.5-fold higher than WT), also consistent with modulation of C-6 substituent binding by M439.

Improved Biocatalytic Breadth of W433C

Valuably, SSβG-WT's very high initial activity at 80° C resulted in enzymes that were still usefully active even after overall reductions in k_{cat}/K_M caused by mutation to E432C and W433C. For example, W433C displays a k_{cat}/K_M towards β-Gal substrates (6.3 $s^{-1}mM^{-1}$) that compares well with the activity of recently described enhanced glycosynthases (k_{cat}/K_M 0.013 $s^{-1}mM^{-1}$) (Mayer *et al., supra*). This activity coupled with greatly broadened specificity resulted in a synthetic utility for W433C and E432C that was demonstrated by the parallel synthesis of β-glycosides of Glc,Gal,Xyl,Man within in a one pot mixture (Scheme 1).



Yield of Products / %

SSβG	Time	1	2	3	4	5	6	7	8
WT	45 min	40	33	2	3	14	8	-	-
WT	4h	-	-	8	20	33	30	4	5
E432C	8h	22	24	14	17	7	9	3	4
W433C	8h	36	18	8	13	11	6	3	5

Scheme 1: Parallel glycoside syntheses using SSβG-WT, -E432C and -W433C as catalysts. The corresponding yields of products (each compound formed is labelled 1-8) are shown in the table. These show that E432C and W433C mutants of SSβG, in which substrate specificity has been tailored, successfully produced balanced libraries of the four, desired β-glycosides of Glc (1), Gal (2), Man (3) and Xyl (4). Such balanced libraries are not produced by SSβG-WT even under varying reaction times.

SSβG-WT was robust enough to catalyze transglycosylation at 50°C in 1:9 MeOH:buffer solutions, to form β-glycosides. However, its stringent specificity meant that after short periods (45 min) only glucoside **1** and galactoside **2** were formed and although small amounts of mannoside **3** and xyloside **4** were observed after extended periods (4h), by this time all initially formed **1** and **2** had been hydrolysed. SSβG-WT is therefore incapable of creating libraries of glycosides in this way. We were therefore delighted to find that both W433C and E432C yielded mixtures of methyl Glc,Gal,Xyl,Man glycosides **1-4**. Indeed, the tailoring of E432C's specificity is so successful that it catalyzes the formation of a small library of **1-4** in which each component is present in near equal amount. This balanced and similar yield of each of **1-4** mirrors the very similar k_{cat} values (2.8-5.4 s^{-1}) of E432C for Glc,Gal,Xyl,Man substrates; an observation that is consistent with the high (> K_M) concentrations of substrates used in these reactions.

Success was achieved in tailoring the specificity of SSβG to create catalysts of broad synthetic utility. The handful of previous examples of substrate specificity alterations in glycosidases have only involved tailoring towards or away from functional groups such as CH_2OH (Zhang *et al., supra* and Andrews *et al., supra*) or phosphate (Teaper *et al.*, Supra). Excitingly, our results suggest that tailoring of stereospecificity is also possible. For example, alteration of a single residue W433→C effectively broadened the Gal:Man stereospecificity 25-fold from 29.4:1 in SSβG-WT to 1.2:1 in SSβG-W433C. Similarly, in the M439C mutant the sum of specificity alteration effects, including a 5-fold absolute increase in Xyl activity, causes a 10-fold increase in Xyl over Fuc specificity. The power of these mutant

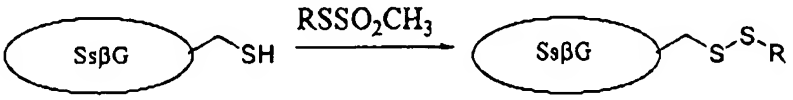
enzymes was further demonstrated by their utility in one-pot parallel syntheses of small arrays of glycosides that could not be accomplished with WT enzyme.

Example 2

1. Summary

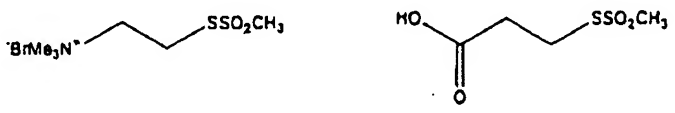
In recent years, chemists have used enzymes such as glycosidases in glycosynthesis (Crout, D. H. G. and Vic, G., *Curr. Opin. Struct. Biol.* 2, 98-111 (1998)), and they are attractive biocatalysts. Research has focussed on site-directed mutagenesis alone as a means of modifying glycosidase activity (Kaper, T., Lebbink, J. H. G., Pouwels, J., Kopp, J., Schulz, G. E., van der Oost, J., and de Vos, W. M., *Biochemistry*, 39, 4963-4970 (2000)), but the construction of mutants is a lengthy process and it is recognised that having a rapid tool for protein modification would be advantageous.

This work, inspired by research conducted on an alkaline protease (Matsumoto, K., Davis, B. G., and Jones, J. B., *Chem. Eur. J.* 8, 4129-4137 (2002)), investigates the combined strategy of site-directed mutagenesis and chemical modification as a means of tailoring the specificity and activity of *Sulfolobus solfataricus* β-glycosidase (SsβG).



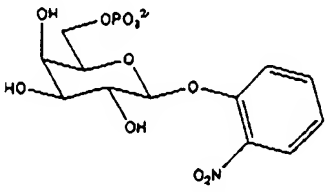
1 2

Chemical modification of a cysteine residue in the active site (1) with methanethiosulfonate reagents (3,4) allowed the facile introduction of functional groups (R) to form mutants (2) with modified electrostatic and steric environments within the active site.



3 4

Modelling of the enzyme active site suggested the synthesis of substrates possessing charged groups at the C-6 hydroxyl (**5**,**6**) to probe the interaction with charged groups present in the chemically modified mutants.



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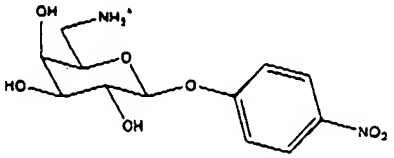


6

The kinetic activity of the mutant enzymes was assessed using ultra-violet/visible spectroscopy and demonstrated that glycosidase activity can be tailored by the combined strategy of site-directed mutagenesis and chemical modification. Initial results suggest that the steric environment of the active site has a greater effect on enzyme activity and specificity than the electrostatic environment.

2. Results and Discussion

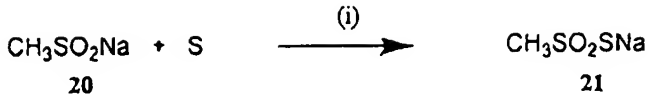
The work falls broadly into three categories – preparation of chemically modified mutants (CMMs) via synthesis of methanethiosulfonate reagents and subsequent chemical modification of C344SM439C, synthesis of substrate molecules and investigation into the kinetics of WT, C344S, C344SM439C and CMMs with various substrates.

2.1 Preparation of Chemically Modified Mutants

2.1.1 Synthesis of methanethiosulfonate reagents

2.1.1.1 Synthesis of sodium methanethiosulfonate

The synthesis of sodium methanethiosulfonate **21**, precursor to functionalised MTS reagents, was achieved by refluxing elemental sulphur and methane sulfinic acid sodium salt **20** in anhydrous methanol (D. Gamblin Part II Thesis, University of Oxford). The insertion reaction proceeded smoothly in a yield of 73% (Scheme 1).



20 21

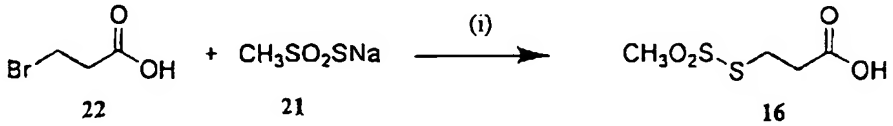
(i) anhydrous methanol, reflux, 20 min, 73%

Scheme 1

5

2.1.1.2 Synthesis of 2-carboxyethyl methanethiosulfonate

This synthesis was achieved following the synthetic route to the analogous 4-carboxybutyl methanethiosulfonate (Davis, B. G., Shang, X., DeSantis, G., Bott, R. R., and Jones, J. B., *Bioorg. Med. Chem.* 7, 2293-2301 (1999)). The reaction
10 proceeded smoothly in a yield of 64% (Scheme 2).



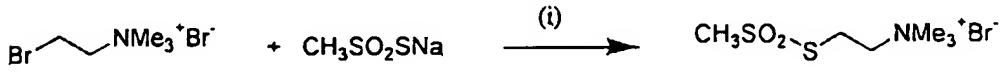
22 21 16

(i) anhydrous DMF, 70°C, 2 h, 64%

15 **Scheme 2**

2.1.1.3 Synthesis of 2-(trimethylammonium)ethyl methanethiosulfonate bromide

Following literature procedure (Davis, B. G., Khumtaveeporn, K., Bott, R. R., and Jones, J. B., *Bioorg. Med. Chem.* 7, 2303-2311 (1999)) the reaction proceeded in
20 a 36% yield (Scheme 3).



23 21 15

(i) anhydrous methanol, reflux, 48 h, 36%

25 **Scheme 3**

2.1.2 Chemical modification of C344SM439C

2.1.2.1 Resuspension of C344SM439C

Chemical modification of C344SM439C was initially attempted using the
30 procedure employed in the chemical modification of SBL which has been developed in our group. However, attempts to resuspend the protein in standard modification

buffer (70 mM CHES, 5 mM MES, 2 mM $CaCl_2$, pH 9.5) were unsuccessful, resulting in protein precipitation. Subsequent Bradford testing (Bradford, M. M., *Anal. Biochem.* 72, 248-254 (1976)) of the protein left in solution showed low protein concentration.

From previous work conducted in the group it was known that WT SsβG and subsequent mutants resuspend well without precipitation in phosphate buffer and show activity therein. Previous kinetic investigtions carried out within the group on SsβG mutants had been conducted at pH 6.5. However, the modification reaction proceeds faster at higher pH values. The upper pH limit of phosphate buffer is pH 9.0 and so this was an imposed limitation on the ligation conditions.
Given these considerations, it was necessary to find a compromise ligation pH value – one which was high enough to encourage rapid modification but which would not be so high as to damage the protein. Accordingly, resuspension of C344SM439C was attempted in phosphate buffer at values of pH 6.24, pp77.68, 8.32 and 8.86 and Bradford testing conducted on the resulting protein solutions.

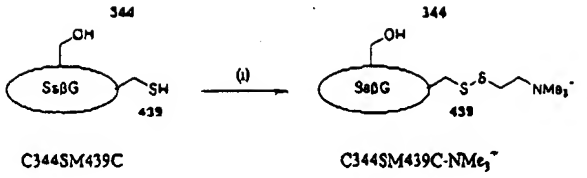
pH	Protein concentration / $mgmL^{-1}$
6.24	0.71
7.68	0.94
8.32	0.84
8.86	0.94

Table 1 : Resuspension of C344SM439C

The Bradford test is only considered accurate to within ~10%, as it relies on the assumption that the test protein will bind to the dye to the same degree as the standard protein, BSA. Table 1 shows the protein concentration determined in each of the resuspension buffers.

2.1.2.2 Chemical modification reaction

To investigate the effect of the ligation pH, the first chemical modification experiment to introduce a trimethyl-ammonium group into position 439 in the active site was carried out at both pH 7.68 and pH 8.86 (Scheme 4).

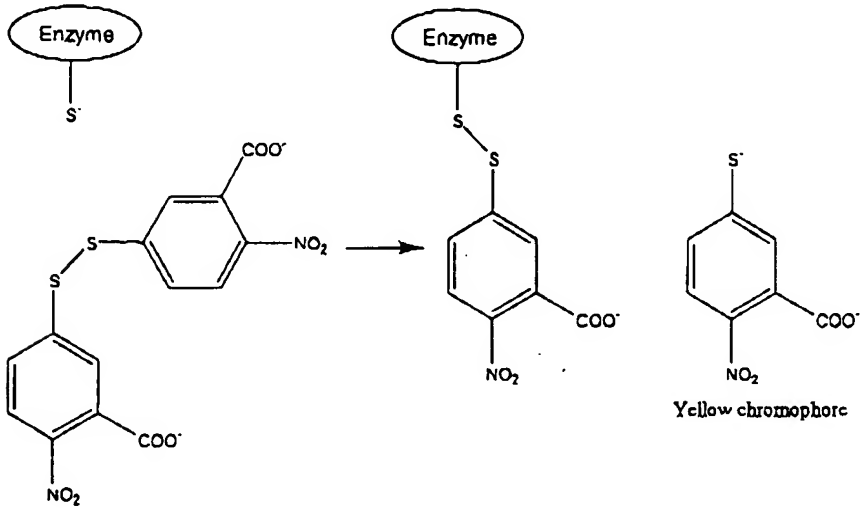


C344SM439C C344SM439C-NMe_3^+

(i) $MeSO_2SCH_2CH_2NMe_3^+Br^-$, ~3 h, pH 7.68 or 8.86

Scheme 4 : Chemical modification

The literature method for monitoring the ligation reaction is by use of Ellman's reagent (Fierobe, H.-P., Mirgorodskaya, E., McGuire, K. A., Roepstorff, P., Svensson, B., and Clarke, A. J., *Biochemistry*, 37, 3743-3752 (1998)), which reacts with free thiols to release a yellow chromophore visible to the naked eye (Scheme 5).



Scheme 5 : Mechanistic action of Ellman's reagent

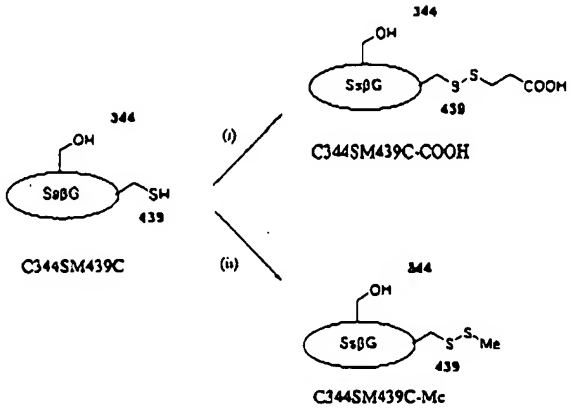
Hence initial testing of an aliquot of colourless reaction mixture with Ellman's reagent should form a yellow solution. As the ligation reaction proceeds subsequent testing should result in the formation of a progressively less yellow solution until finally the aliquot of reaction mixture remains colourless on addition of Ellman's when all the free thiols have reacted with the MTS reagent. Attempts were made to follow the reaction by this method. Prior to the reaction an aliquot of each protein solution was removed for testing with Ellman's reagent. No colour change

was observed, and so sodium hydroxide was added to the mixture to ensure all free thiols would be deprotonated; still no colour change was observed.

Research in the group had shown that by using a solution of Ellman's reagent in ethanol rather than water had made visualisation easier, however, use of this solution resulted in protein precipitation. Attempts were made to measure absorbance at 412 nm (Fierobe, H.-P., Mirgorodskaya, E., McGuire, K. A., Roepstorff, P., Svensson, B., and Clarke, A. J., *Biochemistry*, 37, 3743-3752 (1998)), but the results showed negligible differences between the blank and protein solutions. It was concluded that the protein thiol concentration used in the modification experiment was too low to enable Ellman's reagent to give a conclusive result.

Given these results it was decided to proceed with the ligation reactions without a monitoring method. The reactions were allowed to run for ~3 h. Purification by dialysis and subsequent concentration of solution afforded the CMM in a 59% and 57% yield of recovered protein for the ligation at pH 7.68 and 8.86 respectively. Mass spectrometry showed complete conversion to one product and no remaining starting material in both cases.

Subsequent reactions to produce two other chemically modified mutants were conducted at pH 7.68 (Scheme 6).



(i) MeSO2CH2CH2COOH, ~3 h, pH 7.68
(ii) MeSO2Me, ~3 h, pH 7.68

Scheme 6 : Chemical modification

After 2½ h the excess MTS reagent was removed by centrifugation in Vivaspin concentrators with 10,000 MWCO, as an alternative to dialysis. This purification method afforded the CMMs in higher yields than those achieved for C344SM439C-NMe_3^+. A higher yield of 89% was achieved for C344SM439C-Me and the yield of C344SM439C-COOH was quantitative. Mass spectrometry showed complete conversion to product in both cases. However, initial mass spectra showed high phosphoric acid contamination, which was not removed by drop dialysis. It was believed that phosphoric acid may have been trapped in an enzyme cavity during centrifugation, as unlike dialysis this method of MTS removal does not allow full equilibration between the buffer within the enzyme cavities and the bulk solution. To address this the protein samples were diluted in more buffer and allowed to equilibrate at RT before being prepared for mass spectrometry.

2.1.2.3 Interpretation of mass spectra

All the mass spectra showed the correct mass shift from the reference C344SM439C, to the appropriate CMM (Table 2).

Enzyme	Group introduced	Mass of group	Predicted mass of CMM (based on C344SM439C = 57450)	Found
C344SM439C-NMe_3^+	-$SCH_2CH_2NMe_3^+$	119	57568	57568
C344SM439C-COOH	-SCH_2CH_2COOH	105	57554	57554
C344SM439C-Me	-SMe	47	57496	57496

Table 2 : Mass spectrometry data

It should be noted that the reference mass value of C344SM439C = 57450 does not agree with the literature database value of C344SM439C = 57504. This may be rationalized in two parts. Firstly, N-terminal sequencing of WT SsβG and a mutant within our group has shown that N-terminal methionine residue cleavage occurs during expression resulting in a mass loss of 131 Da, equal to the cleaved residue. Secondly, the use of phosphate buffer creates phosphate adducts in mass spectrometry (Chowdhury, S. K., Katta, V., Beavis, R. C. and Chait, B.T., *J. Am.*

Soc. Mass. Spectrom. 1, 382-388 (1990)). The SsβG protein sample was suspended in phosphate buffer prior to preparation for mass spectrometry. The enzymes were detected as phosphate adducts (phosphate, PO_3^{2-} = 79 Da). These two modifications account well for the observed +77 Da mass difference (at ~ 57500 Da ± 2 Da is an acceptable margin of error, the mass spectra were refined to 2 Da resolution).

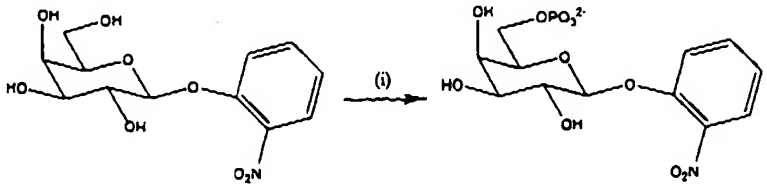
Enzyme	Lit. mass	Lit.mass – Met + PO_3^{2-}	Found	Difference
C344SM439C	57504	57452	57450	2 Da
C344SM439C-NMe_3^+	57622	57570	57568	2 Da
C344SM439C-COOH	57608	57556	57554	2 Da
C344SM439C-Me	57550	57498	57496	2 Da

Table 3 : Mass spectrometry data

2.2 Synthesis of Target Substrates

2.2.1 Synthesis of *o*-nitrophenyl β-D-galactopyranoside-6-phosphate

Treatment of *o*-nitrophenyl β-D-galactopyranoside with trimethyl phosphate and phosphorous oxychloride as a route to *o*-nitrophenyl β-D-galactopyranoside-6-phosphate (Scheme 7) has been described by Hengstenberg, W. and Morse, M. L., *Carbohydrate Res.* **10**, 463-465 (1969).



(i) trimethyl phosphate, phosphorous oxychloride, water, 0°C, 3 h, 62%

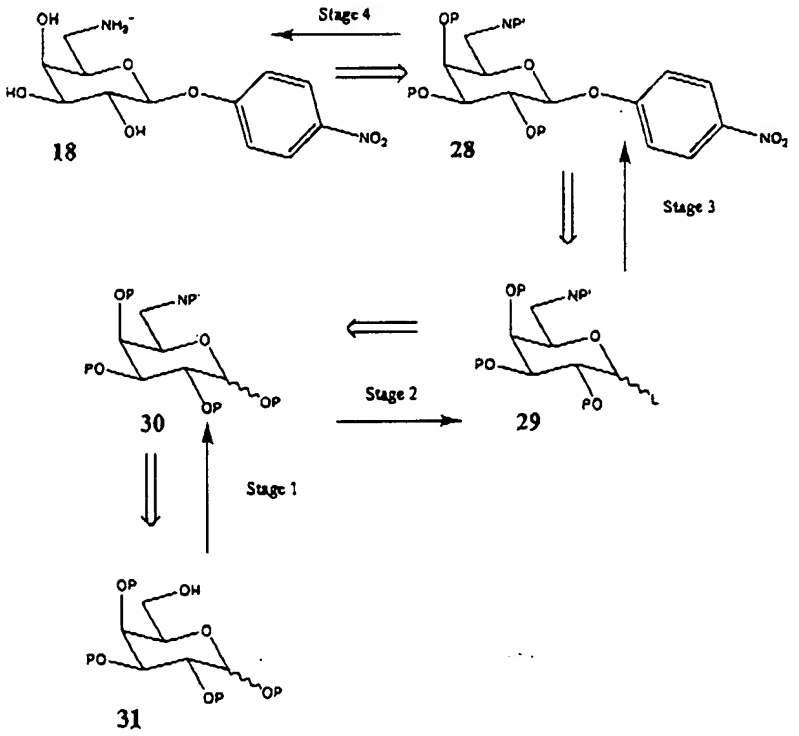
Scheme 7

Neutralization of phosphoric and hydrochloric acids with ammonia solution resulted in the reaction mixture containing inorganic salts in addition to the product,

starting material, β-D-galactopyranose and *o*-nitrophenol resulting from starting material decomposition. The *o*-nitrophenol was removed by co-evaporation with water until the aqueous solution was colourless. To remove the inorganic salts the residue was then absorbed onto acidified charcoal:celite column and eluted with water. The removal of these salts was monitored by reaction of the eluant with silver nitrate solution (the clear solution becomes turbid in the presence of chloride ions), the assumption being made that chloride and phosphate salts would elute at approximately the same rate. Upon complete removal of these inorganic salts *o*-nitrophenyl β-D-galactopyranoside-6-phosphate, *o*-nitrophenyl β-D-galactopyranosideand β-D-galactopyranose were removed from the column by elution with pyridine solution. The product was isolated as the cyclohexylammonium salt in a yield of 62%.

2.2.2 Synthesis of *p*-nitrophenyl 6-amino-6-deoxy-□-D-galactopyranoside

2.2.2.1 Retrosynthetic analysis



Scheme 8 : Retrosynthetic analysis of *p*-nitrophenyl 6-amino-6-deoxy-β-D-galactopyranoside

The synthesis of the target molecule **18** can be separated into four distinct stages. The first stage is to replace the C-6 hydroxyl with a protected nitrogen group (NP') which may be deprotected in later steps to give access to the amine. In order to introduce this regioselectively it is necessary that all other hydroxyl groups are protected (P).

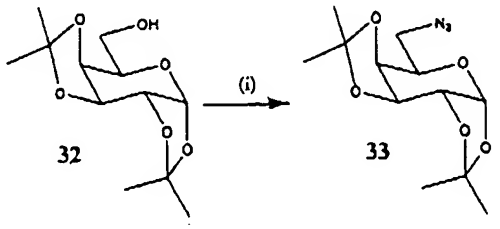
The second stage in the synthesis is to introduce a suitable leaving group (L) at the anomeric position, to then enable stereocontrolled introduction (stage 3) of the chromophore to the anomeric position to give the β-product. In this case, the chromophore selected is *p*-nitrophenol. Once access to **28** is achieved, the fourth remaining stage is to deprotect both the hydroxyl groups and the nitrogen to yield the target molecule **18**.

The nitrogen protecting group selected is an azide and the tetra-protected sugar starting material chosen for this initial step is 1,2:3,4-di*iso*propylidene-α-D-galactopyranose. This is because it is readily available, and direct access to 6-azido-6-deoxy-1,2:3,4-di*iso*propylidene-α-D-galactopyranose can be achieved by use of a modified Mitsunobu reaction.

The resulting sugar can then be de-protected with acid and subsequently re-protected with acetyl protecting groups. The strategy behind this change in protection groups is that the presence of acetyl groups will allow neighbouring group participation to be utilized in future steps to control the anomeric stereochemistry upon chromophore addition. Access to **28** may be achieved via an α-bromide or other leaving group L. The atom introduced at the anomeric position of the tetra-acetyl protected sugar may serve as a leaving group in the following step to introduce the chromophoric group. Activation followed by attack by *p*-nitrophenol should yield exclusively *p*-nitrophenyl 2,3,4-tri-*O*-acetyl-6-azido-6-deoxy-β-D-galactopyranoside. This can then be deprotected by base. The remaining azide deprotection step may normally be achieved by either catalytic hydrogenation or a Staudinger reaction. However, in this particular case catalytic hydrogenation is a less viable option owing to the presence of the aromatic ring and nitro group which might also be hydrogenated, hence deprotection of the azide via a Staudinger reaction will yield the target molecule **18**.

2.2.2.2 Preparation of 6-azido-6-deoxy-di*iso*propylidene-α-D-galactopyranose

Research conducted by Moris-Varas, F., Qian, X.-H., and Wong, C.-H., *J. Am. Chem. Soc.* **118**, 7647-7652 (1996) described the use of a modified Mitsunobu reaction as a means of replacing the 6-position hydroxyl group on a protected sugar with an azide group (Scheme 9).



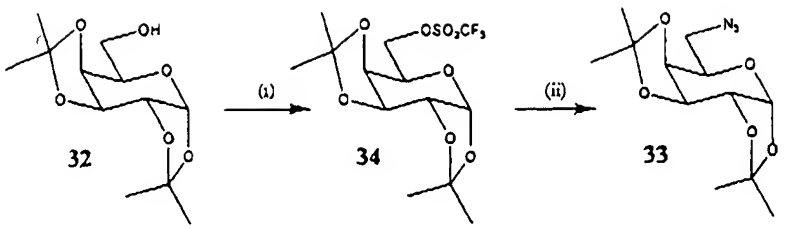
(i) triphenyl phosphine, di*iso*propylazodicarboxylate, hydrazoic acid, toluene, 97%

Scheme 9

Initial attempts at this reaction gave poor yields in the region of 30%, despite t.l.c after 2 h indicating the reaction had seemingly run to completion with formation of one product. However, after basic work up three compounds were visible by t.l.c. Purification by flash column chromatography allowed these to be separated and were shown to be the desired product, starting material and di*iso*propylazodicarboxylate. In subsequent reactions, a micro-work up was performed on an aliquot of the reaction mixture prior to t.l.c. Consequently, the reaction was shown not to have run to completion after 2 h, and accordingly the reaction time was increased with the yield being optimised at 97% after 67 h.

2.2.2.3 Alternative route to 6-azido-6-deoxy-di*iso*propylidene α-D-galactopyranose

Whilst the outcome of the above reaction was investigated an alternative route to 6-azido-6-deoxy-di*iso*propylidene-D-galactopyranose was also evaluated (Scheme 10) (Han, J. W. and Hayashi, T., *Chem. Lett.* **10**, 976-977 (2001)).



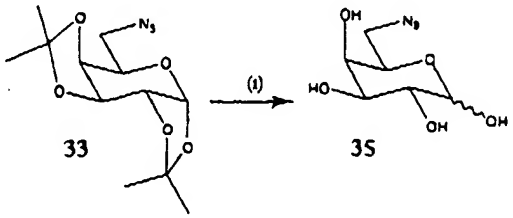
(i) trifluoromethane sulfonic anhydride, pyridine, DCM, 2 h, 42%
(ii) sodium azide, DMF, 23 h, 80%

Scheme 10

1,2:3,4-di*iso*propylidene-α-D-galactopyranose was treated with triflic anhydride and pyridine in DCM to form the primary triflate. Subsequent displacement with sodium azide afforded 6-azido-6-deoxy-di*iso*propylidene-α-D-galactopyranose in a yield of 32% over 2 steps. Although work on this strategy was discontinued after a 97% yield was achieved via the modified Mitsunobu route, it is possible that this two-step yield can be increased if unstable 1,2:3,4-di*iso*propylidene-trifluoromethanesulfonate-α-D-galactopyranose is carried forward without purification and reacted immediately with sodium azide.

2.2.2.4 Preparation of 6-azido-6-deoxy-1,2,3,4-tetra-*O*-acetyl-D-galactopyranose

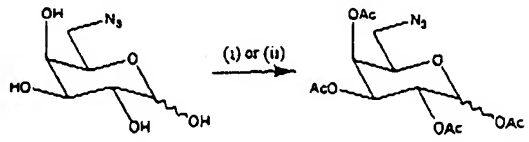
With 6-azido-6-deoxy-di*iso*propylidene-D-galactopyranose in hand, exchange of the *iso*propylidene protecting groups for acetyl protecting groups could take place. The *iso*propylidene groups were removed by aqueous acetic acid at 70°C (Scheme 11) (Moris-Varas, F., Qian, X.-H., and Wong, C.-H., *J. Am. Chem. Soc.* **118**, 7647-7652 (1996)).



(i) acetic acid (aqueous, 80%), 70°C, 69 h, 63%

Scheme 11

The deprotection reaction proceeded smoothly, and in the subsequent reprotection step two methods of acetylation were compared (Scheme 10, Table 4) (Kartha, K. P. R., and Field, R., A., *Tetrahedron*, 53, 11753-11766 (1997)).



35 36

Scheme 12

Method	Reagents	Reaction time	Yield
(i)	acetic anhydride, iodine	5½ h	49%
(ii)	acetic anhydride, pyridine, 4-(dimethylamino)pyridine	75 h	80%

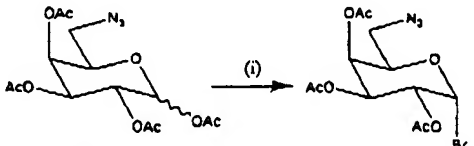
Table 4 : Comparison of acetylation methods

It was decided to use method (ii) as it gave a higher yield. When the de-protection and re-protection steps were conducted consecutively without purification of 35 the yield over two steps was optimised at 90%.

2.2.2.5 Attachment of *p*-nitrophenol at the anomeric centre

2.2.2.5.1 Via 2,3,4-tri-*O*-acetyl-6-azido-6-deoxy-α-D-galactopyranosyl bromide

The acetate **30** was treated with hydrogen bromide in acetic acid to afford **29** which was used without further purification (Scheme 13) (Mitchell, M. B., and Whitcomb, W. A. I., *Tetrahedron Lett.* **41**, 8829-8834 (2000)).

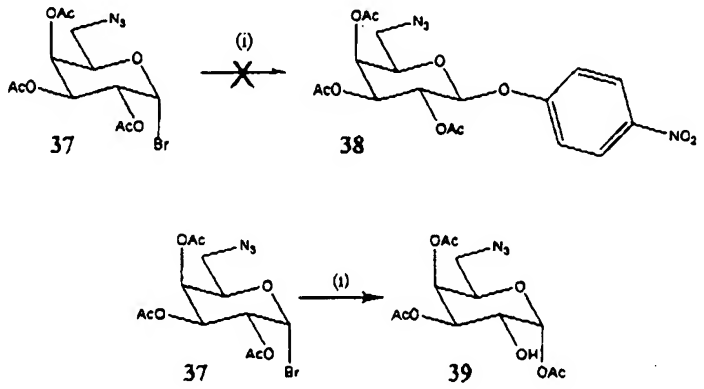


36 37

(i) hydrogen bromide (30% in acetic acid), DCM, 0°C

Scheme 13

The bromide **37** was treated with silver triflate and *p*-nitrophenol in the presence of base (Ottoson, H., *Carbohydrate Res*, **197**, 101-107 (1990)) (Scheme 14). However, none of the expected product **38** was formed, instead acetate migration occurred to give **39**. Surprisingly, characterization of **39** by m/z was not possible.



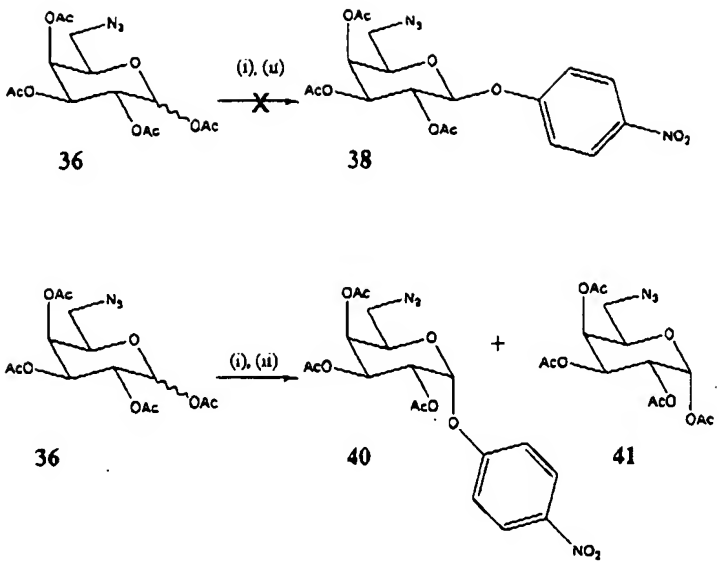
(i) silver triflate, 2, 6-di-tert-butyl-4-methyl-pyridine, *p*-nitrophenol, DCM, molecular sieves, 1 h, 68%

Scheme 14

However other characterization was considered conclusive evidence even in the absence of supportive *m/z* spectra; nmr spectra indicated three acetyl groups, showed an anomeric proton nmr peak at δ 6.37 ppm, typical of the presence of a deshielded acetyl group at the anomeric position and also indicated $J_{1,2}$ = 3.2 Hz which is characteristic of the α-anomer. IR showed absorptions characteristic of C=O and O-H bonds.

2.2.2.5.2 Via direct displacement of acetate with *p*-nitrophenol

After the above method of formation of **38** via the bromide **37** proved to be unsuccessful, attempts were made to attach *p*-nitrophenol using the tetraacetate **36** as a glycosyl donor and Lewis acid cataylsis in DCM (Nishida, Y., Takamori, Y., Matsuda, K., Ohrui, H., Yamada, T., Kobayashi, K., *J. Carb. Chem.* **18**, 985-997 (1999)) to form the desired β-anomer (Scheme 15). This reaction unexpectedly gave the α-anomer **40** rather than the β-anomer **38**.

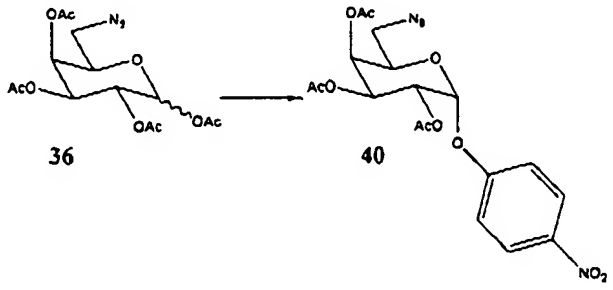


(i) boron trifluoride diethyl etherate, DCM, 20 min

(ii) *p*-nitrophenol, DCM, 65 min, 14%

Scheme 15

Scheme 15 shows the most successful reaction conditions. In initial reactions, all the reactants were mixed together from the start. Under these conditions some product **40** was formed but isolation of a pure sample was not achieved. Reaction conditions were varied in order to optimise yield (Scheme 16 below, Table 5).



Scheme 16 : Reaction to be optimized

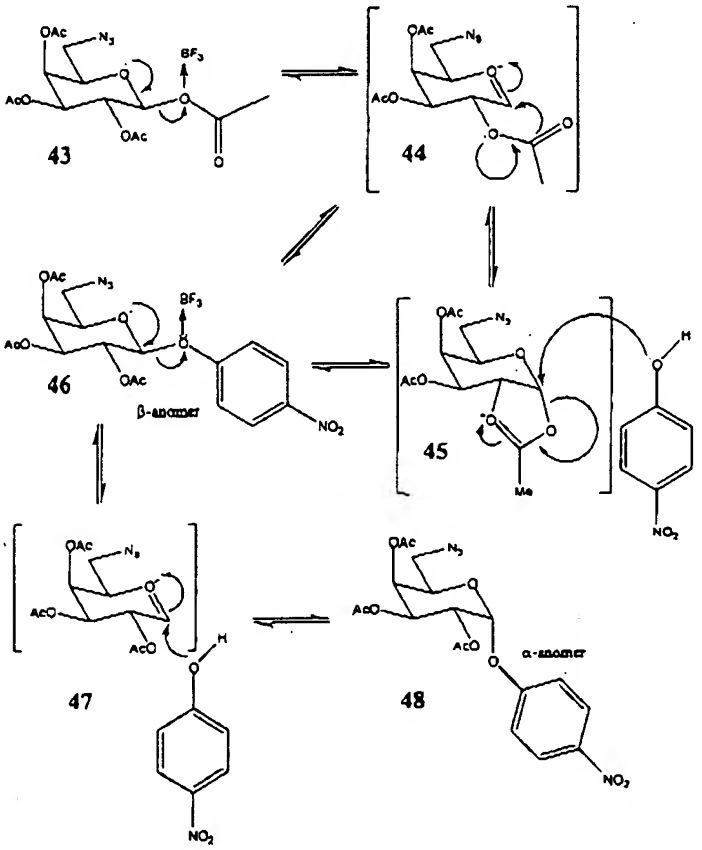
Number of eq of $BF_3.Et_2O$ used	Addition method	Reaction temp	Reaction time	Product 40 yield
1	All reactants together	0°C	1 h	8%
1	Premix 36 and $BF_3.Et_2O$	RT	1 h 40 min	10%
1	All reactants together	RT	2 h	Some product formation, but heavy *p*NP contamination
1	All reactants together	RT	3 h	none
5	All reactants together	RT	50 h	none
5	Premix 36 and $BF_3.Et_2O$	RT	65 min	14%

Table 5 : Reaction conditions for glycosidic bond formation

Monitoring this reaction proved problematic, as the starting material **36** and *p*-nitrophenol co-ran to some extent in all tested t.l.c. solvent systems. *p*-Nitrophenol has a very similar R_f value to that of the product in addition to that of the starting material, therefore purification by flash column chromatography alone was insufficient, and it was necessary to co-evaporate water from the crude product to reduce the amount of *p*-nitrophenol present in the mixture and ease the subsequent purification step. Yields of product were low and a large proportion of material recovered after purification was identified as the α-anomer of the starting material, 6-azido-6-deoxy-1,2,3,4-tetra-*O*-acetyl-α-D-galactopyranose **41**. After extended reaction times (> 2 h) product **40** was not isolated, however, the column fractions with low R_f values did show characteristic azide absorptions in IR and peaks typical of a galactose derivative in nmr spectra. It is suggested that after extended reaction times the Lewis acid may remove the acetyl protecting groups (Askin, D., Angst, C., Danishefsky, S., *J. Org. Chem.* **52**, 622-635 (1987)).

It was discovered that for the product **40** $^3J_{1,2} = 3.7$ Hz, which in the case of galactose is characteristic of the α-anomer. In order to be certain that the α-anomer **40** had been formed, the coupling constant $^1J_{C\text{-}1,H\text{-}1}$ was measured. Bock and Pedersen Bock, K. and Pedersen, C., *J. Chem. Soc, Perkin Trans.s 2*, 293-297 (1974) have described how $^1J_{C\text{-}1,H\text{-}1}$ coupling constants of α-glycosides are found to be ~ 170 Hz, and for β-glycosides ~ 160 Hz. $^1J_{C\text{-}1,H\text{-}1} = 175$ Hz for the product **40**, thus proving the α-anomer had been formed. It was originally expected that this reaction

(scheme 13) would form the β-anomer due to neighbouring group participation by the C-2 acetate, and indeed this was the reason for the choice of acetyl protecting groups. The postulated mechanism for the formation of the α-anomer is that an equilibrium is set up (Scheme17); initially the β-anomer is formed due to neighbouring group participation. The Lewis acid then co-ordinates to the phenyl oxygen atom and removes the *p*-nitrophenyl group with assistance from the ring oxygen lone pair. The *p*-nitrophenyl then re-attaches to the ring in the α-relative configuration, to form the thermodynamically more stable α-anomer due to the anomeric effect.

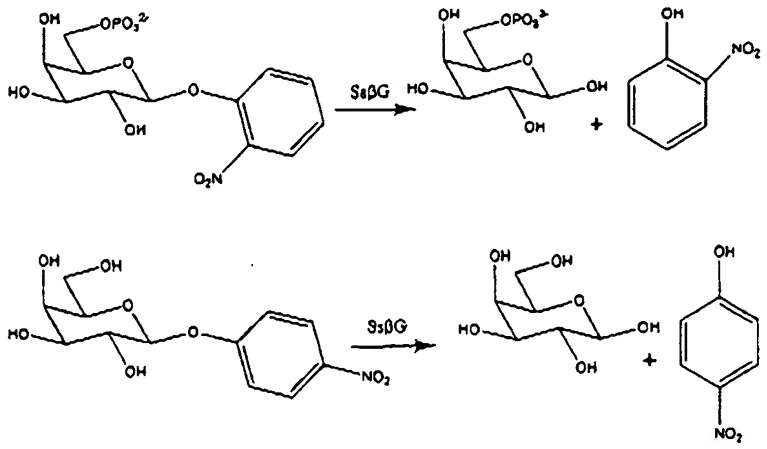


Scheme 17 : Postulated mechanism for Scheme 16

At this point work on this reaction was discontinued as SsβG is β-anomer specific and will not process α-anomers. Unfortunately, time constraints did not allow further investigation into the synthesis of the target molecule **18**.

2.3 Investigation of kinetic parameters

The kinetic activity of SsβG was assessed using ultra-violet/visible spectroscopy. Cleavage of the glycosidic bond of the nitrophenyl sugar analogue releases a chromophore (Scheme 18), either *p*-nitrophenol or *o*-nitrophenol.



Scheme 18: Action of SsβG

The absorbance of these chromophores at 405 nm was continuously measured at regular time intervals, and the Beer-Lambert Law used to calculate the chromophore concentration at each of these time intervals.

$$Abs = \varepsilon cl$$

In order to use this equation the extinction coefficient, ε, of both *p*-nitrophenol and *o*-nitrophenol were calculated. Enzyme kinetic parameters were assessed using the initial rates method. The gradient of a plot of chromophore concentration against time gave the initial rate of reaction at a series of substrate concentrations (0.05-10 mM). Kinetic parameters were calculated by regression analysis of the kinetic data on the Michaelis-Menten Model (Fersht, A., *Enzyme Structure and Mechanism*, W. H. Freeman and Company, New York (1985)). The initial rate of reaction was measured. This model is valid when [substrate] >> [enzyme], which are initial rate conditions.

K_M and v_{max} were calculated from non-linear Michaelis-Menten and linear Lineweaver-Burk plots. From these values, k_{cat} and k_{cat}/K_M were calculated and compared.

$K_M = v_{max}/2$, it is the concentration of substrate at which half the active sites are filled. Higher K_M values correspond to weaker binding and lower K_M values correspond to stronger binding. k_{cat}, the turnover number is the number of substrate molecules which are converted to product when the enzyme is saturated with substrate and v (rate) is maximised. Given both of these considerations, binding and rate, k_{cat}/K_M is typically as a measure of the overall relative activity of the enzyme. To allow comparison of the activity of the enzymes, $\ln(\{k_{cat}/K_M\}\text{mutant}/\{k_{cat}/K_M\}\text{WT})$ was calculated for each enzyme-substrate combination, to give overall activity relative to WT.

2.3.1 Kinetic investigations with *p*-nitrophenyl β-D-galactopyranoside (*p*NPGal)

Figure 2 shows the average value of $\ln(\{k_{cat}/K_M\}\text{mutant}/\{k_{cat}/K_M\}\text{WT})$ over the three runs performed. Positive values indicate higher overall activity relative to WT enzyme, negative values indicate lower overall activity relative to WT.

Enzyme	Side chain structure
WT	$-CH_2CH_2SCH_3$
C344S	$-CH_2CH_2SCH_3$
C344SM439C	-SH
C344SM439C-NMe_3^+	$-SSCH_2CH_2NMe_3^+$
C344SM439C-COOH	$-SSCH_2CH_2COOH$
C344SM439C-Me	$-SSCH_3$

Table 6 : Structure of enzyme side chains at position 439

The two point mutant, C344SM439C, showed highest overall activity of the enzymes screened with *p*NPGal, even greater than the WT enzyme; $\ln(\{k_{cat}/K_M\}\text{mutant}/\{k_{cat}/K_M\}\text{WT})$ is positive. It shows both stronger binding, average $K_M = 376\ \mu M$ vs $K_M = 459\ \mu M$ for WT and a higher average k_{cat}, 18890 s^{-1} compared to 11140 s^{-1} for WT (Table 7).

Enzyme	K_M / mM	Standard deviation	k_{cat} / s^{-1}	Standard deviation	(k_{cat}/K_M) / $M^{-1}s^{-1}$	Standard deviation
WT	0.459	3.92×10^{-2}	5.07	0.48	11140	1780
C344S	0.475	2.52×10^{-2}	4.16	0.17	8787	750
C344SM439C	0.376	1.23×10^{-2}	7.09	0.16	18890	1060
C344SM439C-NMe_3^+	0.464	7.70×10^{-2}	3.48	0.22	7563	706
C344SM439C-COOH*	0.334	2.12×10^{-2}	2.26	0.15	6826	786
C344SM439C-Me	0.272	1.06×10^{-2}	2.29	0.07	8446	86

Table 7 : Kinetic parameters (averaged over 3 runs, except * averaged over 2 runs)

This is further supported by other data generated in the group in which the single point mutant M439C shows higher activity than WT (thanks to Susan Hancock for supplying this data. It is postulated that this is because of the relative steric environments of the active sites. The side chain at position 439 in WT is longer than the side chain in C344SM439C and M439C (Table 6, page 34), the difference of a methionine compared to a cysteine residue, and so there is more space in the active site and less steric hinderance to the incoming substrate.

C344S and the CMMs all show lower overall activity with *p*NPGal compared to WT. Position 344 is not in or near the active site, however, as the C344S mutant shows lower activity than WT, it may be that the mutation causes some alteration in protein structure which in turn alters the structure of the active site and reduces activity. C344SM439C-Me has the highest overall activity of the CMMs, and also has the shortest side chain, causing less steric hinderance to the incoming substrate. C344SM439C-Me and C344SM439C-COOH exhibit lower K_M values than the WT corresponding to stronger binding, but due to their lower k_{cat} values this results in lower overall activity. C344SM439C-NMe_3^+ exhibits weaker bonding with *p*NPGal than the other CMMs, but its higher k_{cat} value leads to similar overall activity.

2.3.2 Kinetic investigations with *o*-nitrophenyl β-D-galactopyranoside-6-phosphate

Having established the kinetic parameters of the six enzymes with *p*NPGal, the same parameters were calculated for the *o*NPGalP6 substrate. The results were similar in that C344SM439C showed the highest overall activity (Figure 3) (for side chain groups see Table 6), and the CMMs on average show slightly lower overall activity compared to WT.

The K_M value (Table 8) for C344SM439C may be explained sterically and electrostatically. The thiol side chain of the cysteine residue in the active site is the smallest of all the enzymes tested, which allows it to best accommodate the bulky phosphate group on the C-6 hydroxyl. Also, hydrogen bonding interactions between the thiol hydrogen and phosphate oxygens may contribute towards increased binding strength.

Enzyme	K_M / mM	Standard deviation	k_{cat} / s^{-1}	Standard deviation	(k_{cat}/K_M) / $M^{-1}s^{-1}$	Standard deviation
WT	2.09	0.32	5.52	0.78	2652	183
C344S	3.59	0.29	8.42	0.21	2357	177
C344SM439C	2.04	0.08	9.11	0.43	4473	188
C344SM439C-NMe_3^+	2.45	0.32	6.21	0.49	2556	319
C344SM439C-COOH*	3.72	0.35	9.40	0.05	2541	225
C344SM439C-Me	4.30	0.30	11.07	0.19	2583	221

Table 8 : Kinetic parameters (averaged over 3 runs, except * averaged over 2 runs)

All of the CMMs have higher k_{cat} values than WT, but also higher K_M values, corresponding to weaker enzyme-substrate binding. However, there is a notable difference between C344SM439C-COOH and C344SM439C-NMe_3^+. The K_M value of C344SM439C-COOH is higher than that of C344SM439C-NMe_3^+ indicating weaker binding. It is possible that this is due to electrostatic repulsion between the carboxylic acid group and the phosphate group, whereas the bonding interaction between the the negatively charged phosphate group and positively charged trimethyl ammonium group is favourable due to their complementary charges.

2.3.3 Comparison of the two data sets

The K_M values for all the enzymes with the phosphorylated substrate are an order of magnitude higher than with the *p*NPGal. There are two postulated reasons for the lower binding strength between *o*NPGalP6 and the enzymes compared to *p*NPGal. Firstly, the phosphate group on the C-6 hydroxyl is larger than the hydroxyl group present in *p*NPGal, and so it is probable that the phosphorylated substrate may encounter greater steric repulsion on entering the active site. The second possibility is that it is due to the relative positioning of the aromatic substituent. The nitro group on the aromatic ring is *ortho* in the case of the phosphorylated substrate, and *para* in

the case of the non-phosphorylated sugar. It may be that the shape of the active site accommodates the *para* group better than the *ortho* group, and hence the former binds more strongly.

For each of the enzymes screened, values of k_{cat} are greater with the phosphorylated substrate than with *p*NPGal, but the higher K_M for *o*NPGalP6-enzyme binding leads to lower overall activity.

2.3.4 Side-chains in the active site

Enzyme	Side-chain	Length of side chain/Å
WT	$-CH_2CH_2SCH_3$	7.28
C344S	$-CH_2CH_2SCH_3$	7.28
C344SM439C	-SH	3.08
C344SM439C-NMe3+	$-SSCH_2CH_2NMe_3^+$	11.34
C344SM439C-COOH	$-SSCH_2CH_2COOH$	10.96
C344SM439C-Me	$-SSCH_3$	6.78

Table 9 : Length of side chains at position 439

Across the six enzymes there are five different side chains present at position 439. Modelling with ChemDraw 3D Pro™ was conducted to elucidate a rough guide to the relative length of these side chains. Each side chain (from the α-carbon) was entered into the programme, its lowest energy configuration obtained by running of MOPAC optimisation and then its bond lengths measured. Obviously, the lowest energy conformation is for the chain 'free in space', not constrained within the environment of a protein active site, in which additional stabilisation/destabilisation forces may affect the exact conformation of the chain. However, treated with appropriate caution, it is believed that this data serves as a rough guide to enable conclusions to be drawn about the effect of the steric bulk of the side chain in the active site.

2.3.5 Summary of kinetics results

All the enzymes screened have shown greater overall activity with *p*NPGal than with *o*NPGalP6, and WT shows the second highest activity with both *p*NPGal and *o*NPGalP6, surpassed only by C344SM439C. The main factor affecting enzyme

activity would appear to be the steric environment of the active site, although the phosphorylated substrate did show a slightly stronger binding affinity with the enzyme possessing a complementary positive charge in the active site.

These results are interesting as it appears that the steric environment of the active site has a greater effect on enzyme activity than the electrostatic environment. At the beginning of this project it was postulated that the charged substrates would show highest activity with enzymes possessing a complementary charge in their active site, increasing binding strength and lowering K_M, and that this would be the major factor affecting enzyme activity. It was also expected that WT would show highest overall activity with *p*NPGal. However, C344SM439C, possessing no charge in the active site and the shortest side chain at position 439 (~ 3 Å), showed the highest activity of all the enzymes with both substrates.

If the steric environment of the active site does not allow the incoming substrate to come within a close enough proximity of any charged groups with which it could have a stabilizing electrostatic interaction, or if any electrostatic interaction causes the positioning of the substrate in the active site to be different to that preferred for optimal performance by the enzyme, then it is possible that no benefit will arise from the modification.

Despite this, these results are encouraging, especially those achieved for the *p*NPGal substrate, as they demonstrate that the enzyme activity can be tailored (in this specific case, lowered) by the combined strategy of site-directed mutagenesis and chemical modification. Comparison of K_M values for C344SM439C-COOH and C344SM439C-NMe_3^+ with *o*NPGalP6 did show stronger binding between the latter and the substrate, indicating that the substrate specificity had been tailored by chemical modification. Further investigations using *p*NPGalP6, and MTS reagents with a shorter chain length may yield more definitive results about the interplay of steric and electrostatic factors in affecting the activity of this enzyme, and hence provide an indication of how best to tailor the substrate specificity.

2.4 Conclusions

The two target MTS reagents (**15**, **16**) were synthesised and used in addition to methyl methanethiolsulfonate to chemically modify C344SM439C. These modifications were confirmed by mass spectrometry. Synthesis of the phosphorylated substrate **17** was successful, and this was used in addition to *p*-nitrophenyl β-D-galactopyranoside **19**, to investigate the kinetic activity of SsβG. These kinetic investigations showed that the activity of SsβG can be tailored by the combined approach of site-directed mutagenesis and chemical modification. However, it appears that the steric environment of the active site has a greater effect on enzyme activity and specificity than the electrostatic environment. Complete synthesis of **18** was not achieved, but access was gained to **36** and **37**, which may provide the basis of future work to complete the synthesis. Further kinetic investigations with other MTS reagents and substrates may yield more definitive results about the interplay of steric and electrostatic factors involved in modifying the activity of SsβG.

3. Experimental

3.1 General Experimental

3.1.1 General synthetic chemistry experimental

Melting points were recorded on a Kofler hot block and are uncorrected. Proton nuclear magnetic resonance (^{1}H) spectra were recorded on Bruker AC 200 (200 MHz), Bruker DPX 400 (400 MHz), Bruker DQX 400 (400 MHz) or by Dr. B. Odell on Bruker AMX 500 (500 MHz) spectrometers. Carbon nuclear magnetic resonance (^{13}C) spectra were recorded on a Bruker DQX 400 (100.6 MHz) or by Dr. B. Odell on Bruker AMX 500 (125.7 MHz) spectrometers. Proton spectra were assigned using COSY. Carbon-13 spectra were assigned using HMQC. Multiplicities were assigned using DEPT or APT sequence. All chemical shifts are quoted on the δ-scale in parts per million (ppm) and are referenced to residual solvent frequencies. Infrared spectra were recorded on a Perkin-Elmer 150 Fourier Transform spectrophotometer. Mass spectra were recorded on a Micromass Platform 1 spectrometer, or by Dr. N. Oldham or Mr. R. Proctor on a Walters 2790-Micromass LCT electrospray ionisation mass spectrometer or Micromass AutoSpec-oaTof

spectrometer and are reported in Daltons and followed by their percentage abundance in parentheses. Optical rotations were measured on a Perkin-Elmer 241 polarimeter with a path length of 1 dm. Concentrations are given in g/100 ml. Thin layer chromatography (t.l.c.) was performed on Merck aluminium backed plates precoated with silica (0.2 mm, 60 F_{254}) or Merck Kieselgel glass-backed sheets pre-coated with silica (0.22-0.25 mm, 60 F_{254}). Plates were visualised using i) ultraviolet lamp (λ_{max} = 254 nm), ii) ninhydrin (0.2% in methanol), iii) phosphomolybdic acid (10% in ethanol), iv) methanol:water:sulphuric acid (conc) 45:45:3. Flash column chromatography was carried out on silica gel (Fluka Kieselgel 60 220-440 mesh) (Still, W. C., Kahn, M., and Mitra, A., *J. Org. Chem.* 43, 2923-2925 (1978)). Solvents and reagents were dried and purified before use; dichloromethane was distilled from calcium hydride, all other anhydrous solvents were purchased directly from manufacturer. 'Petrol' refers to the fraction of light petroleum ether boiling in the range 40-60°C.

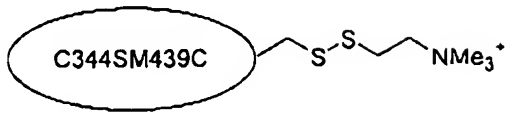
3.1.2 General biological experimental

Sodium phosphate buffer solutions (50 mM) were prepared according to the method described by Gomori using the Henderson-Hasselbalch equation (Sambrook, J., and Russell, D. W., *Molecular Cloning a Laboratory Manual Volume 3*, Cold Spring Harbor Laboratory Press, New York (2001)). Ultra-pure water describes distilled water, de-ionised to 18.2 MΩ resistivity from an Elga Maxima unit coupled to an Elgastat Prima reverse osmosis system. Ammonium acetate buffer describes a 10 mM solution in ultra-pure water pH 6.78. The pH of solutions was measured with a Jenway 3320 pH meter connected to a Gelplas (BDH) electrode. This was calibrated at pH 4.0, 7.0, and 10.0 before use and stored in saturated potassium chloride solution. Centrifugation was performed at room temperature in a MSE Micro Centaur centrifuge at 13,000 r.p.m. Protein mass spectra were recorded on Micromass Platform 2 spectrometer. Absorbance was measured using a Molecular Devices Spectra Max Plus plate reader. Bradford reagent concentrate was purchased from Bio-Rad. Dialysis tubing was purchased from Medicell International Ltd.

3.2 General Biological Procedures

3.2.1 Chemical modification of C344SM439C

C344SM439C-NMe_3^+



C344SM439C (53.1 mg of a lyophilised purified protein sample) was suspended in phosphate buffer (1 mL, 50 mM, pH 7.68) and agitated on a tube rotator. After 15 min, the solution was filtered (0.2 μm Nalgene syringe filter). The filtrate was analysed for protein concentration using the Bradford test (found 0.94 $mgmL^{-1}$) and a portion of this solution (100 μL) was retained for mass spectrometry analysis. A solution of 2-(trimethylammonium)ethyl methanethiosulfonate bromide 15 (1 mg, 4 μmol) in phosphate buffer (200 μL, 50 mM, pH 7.68) was prepared. A portion of this solution (100 μL) was added to the protein solution, mixed by vortexing (5 s) and then agitated on a tube rotator at room temperature. After 30 min, the remainder of the 2-(trimethylammonium)ethyl methanethiosulfonate bromide solution (100 μL) was added, mixed by vortexing (5 s) and then agitated on a tube rotator. After 105 min, the reaction solution was transferred into dialysis tubing. The reaction mixture was dialysed in phosphate buffer (pH 6.42, 50 mM, 1 L, 2 x 1 h). The resulting solution (1500 μL) was concentrated in a Vivaspin 0.5 mL concentrator (10,000 MWCO, pre-washed with ultra-pure water (100 μL), and phosphate buffer (100 μL, 50 mM, pH 6.42)) to a volume of 25 μL (concentrator minimum volume). The solution was diluted with phosphate buffer (975 μL, 50 mM, pH 6.24) to afford a solution of C344SM439C-NMe_3^+ in phosphate buffer (pH 6.42) (0.55 $mgmL^{-1}$, 59%); *m/z* (ES+) 57568 (C344SM439C-NMe_3^+ + covalently bound phosphate, 100%).

C344SM439C-Me and C344SM439C-COOH

C344SM439C (300 mg of a lyophilised purified protein sample) was resuspended in phosphate buffer (6 mL, 50 mM, pH 7.68) and agitated on a tube rotator. After 15 min, the solution was filtered (0.2 μm Nalgene syringe filter). The resulting solution was analysed for protein concentration using the Bradford test (found 0.80 $mgmL^{-1}$). A solution of MTS reagent (1 mg, 5.4 μM, 2-carboxyethyl methanethiosulfonate **16** or 1 μL, 9.7 μM, methyl methanethiolsulfonate) in phosphate buffer (200 μL, 50 mM, pH 7.68) was prepared. A portion of this solution (100 μL) was added to the protein solution, mixed by vortexing (5 s) and then agitated on a tube rotator at room temperature. After 30 min, the remainder of the MTS solution (100 μL) was added, mixed by vortexing (5 s) and then agitated on a tube rotator. After 2 h, the reaction mixture was concentrated in a Vivaspin 0.5 mL concentrator (10,000 MWCO, pre-washed with ultra-pure water (100 μL), and phosphate buffer (100 μL, 50 mM, pH 6.49)) to a volume of 25 μL (concentrator minimum volume). The concentrate was washed with phosphate buffer (4 x 100 μL, 50 mM, pH 6.49) and then diluted with phosphate buffer (975 μL, 50 mM, pH 6.49). An aliquot of this solution (100 μL) was removed and diluted with phosphate buffer (pH 6.5, 50 mM, 1 mL) and agitated on a tube rotator. After 7 h, the solution was concentrated in a Vivaspin 0.5 mL concentrator (10,000 MWCO, pre-washed with ultra-pure water (100 μL), and phosphate buffer (100 μL, 50 mM, pH 6.49)) to a volume of 25 μL (concentrator minimum volume), and then diluted with phosphate buffer (75 μL, 50 mM, pH 6.49) to afford a solution of CMM (100 μL in 50 mM, pH 6.5 phosphate buffer) for mass spectrometry analysis (for preparation see 3.2.2).

C344SM439C-COOH was afforded as a solution in phosphate buffer (50 mM, pH 6.49) (quantitative yield); *m/z* (ES+) 57554 (C344SM439C-COOH + covalently bound phosphate, 100%). C344SM439C-Me was afforded as a solution in phosphate buffer (pH 6.49) (89%); *m/z* (ES+) 57496 (C344SM439C-Me + covalently bound phosphate, 100%).

3.2.2 Bradford test method

Bovine Serum Albumin (BSA) standards in the range of 0.1-1 $mgmL^{-1}$ were prepared from a 10 $mgml^{-1}$ stock solution. Bradford reagent was prepared by 5-fold dilution of dye concentrate with ultra-pure water and then filtration through filter paper under gravity according to manufacturers protocol. In a 96-well flat bottom microtitre plate, Bradford reagent (200 μL) was added to the sample (4 μL) (either blank, BSA standard or test protein) and manually agitated for 5 min before measurement commenced. Protein samples were diluted to ensure $A_{595} < 1$. Measurement of each dilution of the reference protein was conducted in triplicate. Absorbance was measured at 595 nm according to literature protocol (Fierobe, H.-P., Mirgorodskaya, E., McGuire, K. A., Roepstorff, P., Svensson, B., and Clarke, A. J., *Biochemistry*, **37**, 3743-3752 (1998)).

3.2.3 Preparation of protein samples for mass spectrometry

In order to change the buffer, protein solution (100 μL of a ~ 20 μM solution in phosphate buffer) was concentrated in a Vivaspin 0.5 mL concentrator (10,000 MWCO, pre-washed with ultra-pure water (100 μL), and ammonium acetate (100 μL) to a volume of 25 μL (concentrator minimum volume). The concentrate was washed with ammonium acetate (4 x 100 μL) and then diluted with ammonium acetate (75 μL). Mass spectrometry was conducted on this solution. In instances where phosphoric acid contamination was evident, the sample was purified further by drop dialysis; protein solution (10 μL of a ~ 20 μM solution in ammonium acetate as prepared above) was mixed with an acidic solution (water, 5% methanol, 3% formic acid, (10 μL)). A Millipore filter (0.025 μm pore size, 25 mm diameter) was floated in a dish of water and the prepared solution dropped onto the centre of the membrane. After 15 min, the drop was removed and diluted with acetonitrile (20 μL). Mass spectrometry was then conducted on this solution.

3.2.4 Calculation of extinction coefficient of *o*-nitrophenol

o-Nitrophenol (14 mg, 0.10 mmol) was dissolved in phosphate buffer (10 mL, 50 mM, pH 6.49) to give a 10 mM solution. From this stock solution a range of concentrations were prepared (12.5, 25.0, 50.0, 75.0, 100, 1000 μM) by serial

dilution. An aliquot of each *o*-nitrophenol solution concentration and a blank sample of phosphate buffer (300 µL, 50 mM, pH 6.49) was dispensed into seven sealed 1.5 mL Eppendorf tubes. The tubes were incubated in a Techni Dri Block at 45°C. Simultaneously a 96-well flat bottom microtitre plate was incubated at 45°C in a plate reader. After 5 min, 200 µL of each *o*-nitrophenol solution and the phosphate buffer was dispensed into a well in the microtitre plate. The plate containing the solutions and buffer was then incubated in the plate reader at 45°C. After 5 min the absorbance at 405 nm was measured. A straight line graph of absorption against concentration gave a gradient equal to the extinction coefficient according to Beer-Lambert Law.

3.2.5 Kinetic assays

Substrate solutions (concentration 10 mM in 50 mM pH 6.5 phosphate buffer) were prepared. Kinetic assays were conducted in a 96-well flat bottom microtitre plate. Eight substrate concentrations were chosen for the assay from the range 0.05 mM to 10 mM (prepared from 10 mM stock solution), based on previous experimental experience of the kinetics of each enzyme (default range 0.05 mM to 2.00 mM)*. The enzyme stock solution (~ 1 $mgmL^{-1}$) was diluted between 16- and 80-fold depending on the kinetic parameters determined*. The enzyme solution (496 □L) was dispensed into a 1.5 mL sealed Eppendorf tube. Into eight further Eppendorf tubes a portion of each substrate solution (650 µL) was dispensed. The tubes were incubated in a Techni Dri Block at 45°C. Simultaneously a 96-well flat bottom microtitre plate was incubated at 45°C in a plate reader. After 5 min, substrate (190 µL) was dispensed into the microtitre plate in triplicate and 24 aliquots of of the enzyme solution (15 µL) were dispensed into the plate. The plate containing the enzyme and substrate solutions was then incubated in the plate reader at 45°C to allow equilibration. After 5 min, enzyme solution (10 µL) was added to each well containing substrate solution to initiate the reaction and the data collection commenced. Release of *p*-nitrophenol/*o*-nitrophenol was measured by absorbance at 405 nm, with an automix of 3 s before the first read and 1 s between every subsequent read. The run time chosen was between 6 min and 10 min*, and readings were taken at intervals of between 6 s and 10 s*.

* See Table 10 for specific substrate solutions, enzyme concentrations, run times and intervals used in each experiment.

Expt	Enzyme	[Enzyme]/ mM	Substrate	[Substrate] Used/mM	Run t/min	Interval/s
1	C344S	3.7×10^{-5}	*p*NPGal	0.10, 0.25, 0.50, 0.75, 1.00, 1.50, 2.00, 5.00.	10	10
2	C344SM439C	1.9×10^{-5}	*p*NPGal	0.25, 0.50, 0.75, 1.00, 1.50, 2.00, 5.00, 10.00.	6	6
3	C344SM439C-Me	3.9×10^{-5}	*p*NPGal	0.10, 0.25, 0.50, 0.75, 1.00, 1.50, 2.00, 5.00.	10	10
4	C344SM439C-NMe_3^+	3.0×10^{-5}	*p*NPGal	0.10, 0.25, 0.50, 0.75, 1.00, 1.50, 2.00, 5.00.	10	10
5	C344SM439C-COOH	4.6×10^{-5}	*p*NPGal	0.10, 0.25, 0.50, 0.75, 1.00, 1.50, 2.00, 5.00.	10	10
6	WT	2.3×10^{-5}	*o*NPGalP6	0.05, 0.10, 0.2, 0.50, 0.75, 1.00, 1.50, 2.00.	10	10
7	C344S	1.5×10^{-5}	*o*NPGalP6	0.05, 0.10, 0.2, 0.50, 0.75, 1.00, 1.50, 2.00.	10	10
8	C344SM439C	1.0×10^{-5}	*o*NPGalP6	0.05, 0.10, 0.2, 0.50, 0.75, 1.00, 1.50, 2.00.	10	10
9	C344SM439C-Me	1.9×10^{-5}	*o*NPGalP6	0.10, 0.25, 0.50, 0.75, 1.00, 1.50, 2.00, 5.00.	10	10
10	C344SM439C-NMe_3^+	1.5×10^{-5}	*o*NPGalP6	0.10, 0.25, 0.50, 0.75, 1.00, 1.50, 2.00, 5.00.	10	10
11	C344SM439C-COOH	4.6×10^{-5}	*o*NPGalP6	0.10, 0.25, 0.50, 0.75, 1.00, 1.50, 2.00, 5.00.	10	10

Table 10 : Kinetic assay experimental details

3.2.6 Kinetic assay data manipulation

A graph of concentration of the released chromophore (either *p*NP or *o*NP) against time was drawn for each concentration using Microsoft Excel. When substrate concentration becomes limiting, the plot fails to produce a straight line. The data up to this point was used to calculate the rate of chromophore release. These gradients were entered into Grafit, which calculated K_M and v_{max} from the Michelis-Menten and Lineweaver-Burk plots. From these k_{cat} and k_{cat}/K_M could be calculated. To compare activities of the different enzymes column charts of $\ln(\{k_{cat}/K_M\}\text{mutant}/\{k_{cat}/K_M\}\text{WT})$ were constructed.

3.2.7 Background substrate degradation determination

Substrate (1 mL of a 10 mM solution) was dispensed into a sealed 1.5 mL Eppendorf tube. The tube was incubated in a Techni Dri Block at 45°C. Simultaneously a 96-well flat bottom microtitre plate was incubated at 45°C in a plate reader. After 5 min, substrate solution (100 μL) was dispensed into a well in the microtitre plate. The plate containing the substrate solution was then incubated in the plate reader at 45°C. After 5 min the absorbance at 405 nm was measured. The solution continued to be incubated in the Techni Dri Block for 70 h, and further measurements were taken at various time intervals. Before each measurement the plate containing substrate solution was incubated in the plate reader at 45°C for 5 min.

3.3 Procedures

Sodium methanethiosulfonate 21

A mixture of methane sulfinic acid sodium salt **20** (2.50 g, 24.5 mmol) and sulfur (784 mg, 24.5 mmol) in methanol (150 mL) was heated to reflux under argon. After 20 min, the sulfur had dissolved and the hot solution was filtered. The filtrate was concentrated *in vacuo* to afford a white solid which was washed with anhydrous ethanol (30 mL) and dried *in vacuo* to afford sodium methanethiosulfonate **21** (2.40 g, 73%) as a white crystalline solid; m.p. 271-272°C (ethanol) [Lit. 272-273.5°C];[21] ν_{max} (thin film) 1323, 1085 (S-SO_2)cm^{-1}; δ_H(200 MHz, D_2O) 3.26 (3H, s, CH_3).

2-Carboxyethyl methanethiosulfonate 16

A solution of 3-bromopropionic acid **22** (571 mg, 3.73 mmol) and sodium methanethiosulfonate **21** (511 mg, 3.81 mmol) in DMF (5 mL) was stirred under argon at 70°C. After 2 h, t.l.c. (ethyl acetate:methanol, 4:1) indicated the formation of two products (R_f 0.3, 0.6) and the absence of any starting material (R_f 0.2). The reaction mixture was cooled to room temperature, water (10 mL) was added and the resulting mixture extracted with ether (3 x 20 mL). The organic extracts were combined, washed with brine (30 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (DCM:ether, 3:1

(acetic acid, 0.6%)) to yield 2-carboxyethyl methanethiosulfonate **16** (438 mg, 64%) as a white crystalline solid; m.p. 44-46°C (ethyl acetate/petrol) [Lit. 44-48°C m.p., value obtained from Toronto Research Chemicals Inc. website (www.trc-canada.com), nmr spectra assigned using Chemical Concepts SpecInfo]; ν_{max} (thin film) 1716 (st, C=O), 1312, 1130 (S-SO_2)cm^{-1}; δ_H (400 MHz, $CDCl_3$) 2.94 (2H, t, $J_{1,2}$ 6.7 Hz, 2 x H-2), 3.36 (3H, s, CH_3), 3.38 (2H, t, 2 x H-1); δ_C (100.6 MHz, $CDCl_3$) 30.6 (t, C-1), 34.4 (t, C-2), 50.6 (q, CH_3), 176.7 (s, C=O); *m/z* (ES-) 183 ($M-H^+$, 100%). (HRMS (ES-) Calcd. For $C_4H_7O_4S_2$ ($M-H^-$) 182.9786. Found 182.9788).

2-(Trimethylammonium)ethyl methanethiosulfonate bromide 15

A solution of sodium methane thiosulfonate **21** (472 mg, 3.52 mmol) and 2-bromoethyltrimethylammonium bromide **23** (838 mg, 3.39 mmol) in anhydrous methanol (7 mL) was heated to reflux under argon. After 48 h, t.l.c (ethyl acetate:methanol, 4:1) indicated formation of one product (R_f 0.0) along with some remaining starting material (R_f 0.4). The solution was cooled to -78°C, then immediately allowed to warm to -15°C. The white precipitate thus formed was filtered and dried *in vacuo* to afford 2-(trimethylammonium)ethyl methanethiosulfonate bromide **15** (356 mg, 36%) as a white crystalline solid; m.p. 155.5-156.5oC (ethanol/ether) [Lit. 157.5-158.5°C (ethanol)])Davis, B. G., Khumtaveeporn, K., Bott, R. R., and Jones, J. B., *Bioorg. Med. Chem.* 7, 2303-2311 (1999)); ν_{max} (thin film) 1317, 1132 (S-SO_2)cm^{-1};δ_H (400 MHz, D_2O) 3.09 (9H, s, $N(CH_3)_3$), 3.47 (3H, s, CH_3SO_2), 3.52-3.55 (2H, m, 2 x H-1), 3.64-3.68 (2H, m, 2 x H-2).

6-Azido-6-deoxy-1,2:3,4-di*iso*propylidene-α-D-galactopyranose 33

Toluene (10 mL) was added to a stirred suspension of sodium azide (2.60 g, 40.0 mmol) in water (2 mL). The reaction mixture was cooled to 5°C and sulfuric acid (1.0 mL, 20.0 mmol) added dropwise. The reaction mixture was stirred under argon at 5°C for 40 min. The organic layer was removed by syringe and dried (Na_2SO_4). The hydrazoic acid thus formed was standardised against potassium hydroxide (0.072 M aqueous solution). Triphenyl phosphine (2.53 g, 9.63 mmol)

was dissolved in toluene (20 mL) and di*iso*propyl azodicarboxylate (1.9 mL, 9.63 mmol) added. The reaction mixture was stirred under argon for 10 min then added to a flask containing a solution of 1,2:3,4-di*iso*propylidene-α-D-galactopyranose **32** (1.00 g, 3.85 mmol) and hydrazoic acid (11.3 mL of a 0.85 M solution in toluene, 9.63 mmol) in toluene (20 mL). After 67 h t.l.c. (petrol:ethyl acetate, 2:1) indicated the formation of a major product (R_f 0.5) and the absence of starting material (R_f 0.2). The reaction mixture was diluted with ether (50 mL), washed with sodium bicarbonate (3 x 50 mL of a saturated aqueous solution), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol:ethyl acetate, 2:1) to afford 6-azido-6-deoxy-1,2:3,4-di*iso*propylidene-α-D-galactopyranose **33** (1.09 g, 97%) as a pale orange oil; $[\alpha]_D^{25}$ –68.7 (*c*, 0.8 in $CHCl_3$) [Lit. $[\alpha]_D^{21}$ –92.1 (*c*, 1.48 in $CHCl_3$ containing 0.75% EtOH)] (Szarek, W. A. and Jones, J. K. N., *Can. J. Chem.* **43**, 2345-56 (1965))]; ν_{max} (thin film) 2102 (sh, N_3)cm^{-1}; δ_H (400 MHz, $CDCl_3$) 1.34, 1.35, 1.46, 1.55 (12H, 4 x s, 4 x CH_3), 3.37 (1H, dd, $J_{5,6}$ 5.4 Hz, $J_{6,6'}$ 12.6 Hz, H-6), 3.52 (1H, dd, $J_{5,6'}$ 7.9 Hz, H-6'), 3.90-3.94 (1H, m, H-5), 4.20 (1H, dd, $J_{3,4}$ 7.9 Hz, $J_{4,5}$ 1.9 Hz, H-4), 4.34 (1H, dd, $J_{1,2}$ 5.1 Hz, $J_{2,3}$ 2.5 Hz, H-2), 4.64 (1H, dd, H-3), 5.55 (1H, d, H-1). Alternative synthesis of 6-azido-6-deoxy-1,2:3,4-di*iso*propylidene-α-D-galactopyranose **33**. A solution of sodium azide (33 mg, 0.51 mmol) in DMF (5 mL) was added to a solution of 1,2:3,4-di*iso*propylidene-6-trifluoromethanesulfonate-α-D-galactopyranose **34** (96 mg, 0.24 mmol) in DMF (5 mL). The reaction mixture was stirred under argon at room temperature. After 19 h, the reaction mixture was heated to 45°C. After 23 h, t.l.c. (petrol:ethyl acetate, 2:1) showed a single spot since the major product (R_f 0.6) co-ran with the starting material. The DMF was removed *in vacuo*. The residue was dissolved in DCM (100 mL), neutralised with sodium bicarbonate (100 mL), washed with brine (3 x 30 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol:ethyl acetate, 2:1) to afford 6-azido-6-deoxy-1,2:3,4-di*iso*propylidene-α-D-galactopyranose **33** (70 mg, 80%) as a pale orange oil identical to that previously described.

1,2:3,4-Di*iso*propylidene-6-trifluoromethanesulfonate-α-D-galactopyranose 34

1,2:3,4-di*iso*propylidene-α-D-galactopyranose **32** (1.05 g, 4.04 mmol) was dissolved in dichloromethane (15 mL). Pyridine (470 µl, 5.77 mmol) and trifluoromethane sulfonic anhydride (710 µl, 4.23 mmol) were added. The reaction mixture was stirred under argon. After 2 h, t.l.c. (petrol:ethyl acetate, 2:1) indicated the formation of two products (R_f 0.1, 0.6) and the absence of any starting material (R_f 0.2). The reaction mixture was diluted with dichloromethane (50 mL), washed with sodium bicarbonate (4 x 30 mL of a saturated aqueous solution), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol:ethyl acetate, 2:1) to afford 1,2:3,4-di*iso*propylidene-trifluoromethanesulfonate-α-D-galactopyranose **34** (0.66 g, 42%) as a pale pink solid R_f 0.6 (petrol:ethyl acetate, 2:1); m.p. 47.7-48.1°C (ethanol/ether) [Lit. 48.5-50.0°C (hexane)] (Barrette, E. P. and Goodman, L., *J Org. Chem.* **49**, 176-178 (1984))]; $[\alpha]_D^{25}$ -42.6 (c, 0.7 in $CDCl_3$) [Lit. $[\alpha]_D^{27}$ –49.9 (*c*, 1.48 in $CHCl_3$)]; ν_{max} (thin film) 1415, 1206 (s, SO_2)cm^{-1}; δ_H (400 MHz, CD_3OD) 1.33, 1.34, 1.41, 1.50 (12H, 4 x s, 4 x CH_3), 4.13-4.16 (1H, m, H-5), 4.29 (1H, dd, $J_{3,4}$ 7.8 Hz, $J_{4,5}$ 2.0 Hz, H-4), 4.41 (1H, dd, $J_{1,2}$ 4.9 Hz, $J_{2,3}$ 2.7 Hz, H-2), 4.57 (1H, dd, $J_{5,6}$ 8.5 Hz, $J_{6,6'}$ 10.8 Hz, H-6), 4.68 (1H, dd, H-3), 4.75 (1H, dd, $J_{5,6'}$ 3.2 Hz, H-6'), 5.51 (1H, d, H-1).

6-Azido-6-deoxy-D-galactopyranose 35

6-azido-6-deoxy-1,2:3,4-di*iso*propylidene-α-D-galactopyranose **33** (100 mg, 0.35 mmol) was dissolved in acetic acid (5 mL of an 80% by volume aqueous solution). The reaction mixture was stirred at 70°C. After 69 h, t.l.c. (petrol:ethyl acetate, 2:1) indicated the formation of a major product (R_f 0.0) and the absence of any starting material (R_f 0.6). The ethanoic acid was removed *in vacuo*. The residue was purified by flash column chromatography (ethyl acetate:methanol, 4:1) to afford 6-azido-6-deoxy-D-galactopyranose **35** (α:β, 1:1) (57 mg, 63%) as a white crystalline solid R_f 0.5 (ethyl acetate:methanol, 4:1); m.p. 58.0-60.0 C (ethanol/ether); $[\alpha]_D^{25}$ +86.0 (*c*, 0.5 in H_2O);ν_{max} (thin film) 3310 (br, OH), 2117 (sh, N_3)cm^{-1}; δ_H (400 MHz, CD_3OD) 3.35-3.41 (3H, m, α-H-6, β-H-3, β-H-6), 3.48-3.58 (4H, m, α-H-3, α-H-6', β-H-2, β-H-6'), 3.70-3.83 (4H, m, α-H-2, α-H-4, β-H-3,

β-H-4), 4.09-4.16 (2H, m, α-H-5, β-H-5), 4.48 (1H, d, $J_{\beta 1,2}$ 7.4 Hz, β-H-1), 5.18 (1H, d, $J_{\alpha 1,2}$ 3.6 Hz, α-H-1).

6-Azido-6-deoxy-1,2,3,4-tetra-*O*-acetyl-D-galactopyranose 36

4-(Dimethylamino)pyridine (1 mg, 0.01 mmol) and pyridine (4.3 mL, 55 mmol) were added to a stirred suspension of 6-azido-6-deoxy-D-galactopyranose **35** (2.24 g, 10.9 mmol) in acetic anhydride (5.2 mL, 54.65 mmol). The reaction mixture was stirred at RT. After 75 h, t.l.c. (petrol:ethyl acteate, 2:1) indicated the formation of one product (R_f 0.4) and the absence of any starting material (R_f 0.0). The reaction mixture was diluted with DCM (150 mL), neutralised with sodium bicarbonate (3 x 100 mL of a saturated aqueous solution), washed with brine (100 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol:ethyl acetate, 2:1) to afford 6-azido-6-deoxy-1,2,3,4-tetra-*O*-acetyl-D-galactopyranose **36** (α:β, 0.8:1) (3.26 g, 80%) as a colourless oil; $[\alpha]_D^{25}$ +49.5 (c, 0.9 in $CDCl_3$); ν_{max} (thin film) 2106 (sh, N_3), 1748 (st, C=O)cm^{-1}; δ_H (500 MHz, $CDCl_3$) 1.99-2.19 (24H, m, 8 x CH_3), 3.46-3.67 (4H, m, α-H-6, α-H06', β-H-6, β-H-6'), 3.82 (1H, at, β-H-5), 4.10-4.14 (2H, m, α-H-5, β-H-4), 5.04 (1H, dd, $J_{\beta 2,3}$ 10.2 Hz, $J_{\beta 3,4}$ 3.2 Hz, β-H-3), 5.30-5.45 (4H, m, α-H-2, α-H-3, α-H-4, β-H-2), 5.69 (1H, d, $J_{\beta 1,2}$ 8.2 Hz, β-H-1), 6.37 (1H, d, $J_{\alpha 1,2}$ 3.9 Hz, α-H-1). Alternate synthesis of 6-azido-6-deoxy-1,2,3,4-tetra-*O*-acetyl-D-galactopyranose **36.** A warmed portion of iodine (10 mg, 0.04 mmol) in acetic anhydride (5 ml, 53 mmol) was added to a stirred suspension of 6-azido-6-deoxy-D-galactopyranose **35** (204 mg, 1.0 mmol) in acetic anhydride (5 ml, 53 mmol), and the reaction mixture was cooled in ice. After 5 min, the reaction mixture was allowed to warm to RT. After 5½ h, t.l.c (petrol:ethyl acetate, 2:1) indicated the formation of several products (R_f 0.2-0.4) and the absence of any starting material (R_f 0.0). The reaction mixture was diluted with DCM (50 mL), washed with sodium thiosulfate (50 mL of a 10% aqueous solution), neutralized with sodium bicarbonate (6 x 100 mL of a saturated aqueous solution), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol:ethyl acetate, 2:1) to afford 6-azido-6-deoxy-1,2,3,4-tetra-*O*-acetyl-D-galactopyranose **36** (α:β, 1:1) (182 mg, 49%) R_f 0.2 (petrol:ethyl acetate, 2:1) as a

colourless oil identical to that previously described In a subsequent reaction 1,2,3,4-tetra-*O*-acetyl-α-D-galactopyranose was isolated as a white crystalline solid; m.p. 89.9-90.3°Ç (ethanol/ether) [Lit. 90°C (ethanol) (Jezo, I. and Zemek, J., *Chemicke Zvesti*, 33, 533-541 (1979)]; $[\alpha]_D^{25}$ +63.4 (*c*, 0.4 in $CHCl_3$) [Lit. $[\alpha]_D^{23}$ +97 (*c*, 1 in $CHCl_3$)]; ν_{max} (thin film) 2105 (sh, N_3), 1642 (st, C=O)cm^{-1}; δ_H (400 MHz, $CDCl_3$) 2.00, 2.01, 2.02, 2.03 (12H, 4 x s, 4 x CH_3), 3.28 (1H, dd, $J_{5,6}$ 5.7 Hz, $J_{6,6'}$ 12.8 Hz, H-6), 3.45 (1H, dd, $J_{5,6'}$ 7.5 Hz, H-6'), 4.24 (1H, m, H-5), 5.35 (2H, m, H-2, H-3), 5.49 (1H, d, H-4), 6.41 (1H, br, H-1).

2,3,4-Tri-*O*-acetyl-6-azido-6-deoxy-α-D-galactopyranosyl bromide 37

Hydrogen bromide (2 mL of a 30% solution in acetic acid) was added to a solution of 6-azido-6-deoxy-1,2,3,4-tetra-*O*-acetyl-galactopyranose **36** (α:β, 0.8:1) (320 mg, 0.86 mmol) in anhydrous DCM (10 mL). The mixture was stirred under argon at 0°C. After 1¾ h, t.l.c. (petrol:ethyl acetate, 2:1) indicated the formation of two products (R_f 0.5, 0.2) with some remaining starting material (R_f 0.3). The reaction mixture was quenched with ice/water (30 mL), diluted with DCM (40 mL), neutralized with sodium bicarbonate (2 x 40 mL), washed with brine (40 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo* to yield 350 mg of crude product, which was used without further purification, but a small portion was retained and purified by flash column chromatography (DCM:ether, 60:1) to afford 2,3,4-tri-*O*-acetyl-6-azido-6-deoxy-α-D-galactopyranosylbromide **37** as a white solid R_f 0.5 (60:1, DCM:ether); m.p. 81.5-82.2C (ether/petrol) [Lit. 82-83°C (ether/petrol) Jezo, I. and Zemek, J., *Chemicke Zvesti*, 33, 533-541 (1979)]; $[\alpha]_D^{25}$ +70.8 (*c*, 1.7 in $CHCl_3$) [Lit. $[\alpha]_D^{22}$ +133.8 (*c*, 1 in $CHCl_3$)]; ν_{max} (thin film) 2107 (sh, N_3), 1750 (st, C=O)cm^{-1}; δ_H (400 MHz, $CDCl_3$) 2.03, 2.12, 2,18 (9H, 3 x s, 3 x CH_3), 3.30-3.40 (2H, m, H-6, H-6'), 4.47 (1H, t, H-5), 5.04 (1H, dd, $J_{1,2}$ 4.0, $J_{2,3}$ 10.7, H-2), 5.43 (1H, dd, $J_{3,4}$ 3.2, H-3), 5.69 (1H, m, H-4), 6.69 (1H, d, H-1).

1,3,4-Tri-*O*-acetyl-6-azido-6-deoxy-2-hydroxy-α-D-galactopyranose 39

2,3,4-tri-*O*-acetyl-6-azido-6-deoxy-α-D-galactopyranosylbromide **37** (100 mg, 0.25 mmol) and *p*-nitrophenol (37 mg, 0.27 mmol) were dissolved in DCM. This solution was added to a stirred suspension of 2,6-di-*tert*-butyl-4-methyl-

pyridine (37 mg, 0.18 mmol), silver triflate (87 mg, 0.30mmol) and molecular sieves (3Å) in DCM (7 ml). The reaction mixture was stirred under argon. After 1 h, t.l.c. (petrol:ethyl acetate, 2:1) indicated complete consumption of starting material (R_f 0.4). The reaction mixture was filtered through celite, concentrated *in vacuo* and co-evaporated with water. The residue was purified by flash column chromatography (DCM:ether, 30:1) to afford 1,3,4-tri-*O*-acetyl-6-azido-6-deoxy-2-hydroxy-α-D-galactopyranose **39** (55 mg, 65%) as a colourless oil (R_f 0.5); partial data $[\alpha]_D^{25}$ +68.7 (*c*, 0.2 in $CHCl_3$); ν_{max} (thin film) 3432 (br, OH), 2101 (sh, N_3) 1644 (st, C=O)cm^{-1}; δ_H (400 MHz, $CDCl_3$) 2.02, 2.03, 2.17, 2.18 (12H, 4 x s, 4 x CH_3), 3.28 (1H, dd, $J_{5,6}$ 7.8 Hz, $J_{6,6'}$ 10.3 Hz, H-6), 3.35 (1H, dd, $J_{5,6'}$ 6.2 Hz, H-6'), 4.31 (1H, m, H-5), 5.32 (1H, dd, $J_{1,2}$ 3.2 Hz, $J_{2,3}$ 11.0 Hz, H-2), 5.37 (1H, dd, $J_{3,4}$ 3.1 Hz, H-3), 5.69-5.70 (1H, m, H-4), 6.37 (1H, d, H-1); δ_C (100.6 MHz, $CDCl_3$) 20.5, 20.6, 20.9 (3 x q, 3 x CH_3), 27.4 (t, C-6), 66.2 (d, C-2), 67.5 (d, C-3), 67.7 (d, C-4), 71.2 (d, C-5), 89.6 (d, C-1), 168.9, 169.9, 170.1 (3 x s, 3 x C=O).

_p_-Nitrophenyl 2,3,4-tri-*O*-acetyl-6-azido-6-deoxy-α-D-galactopyranoside 40

Boron trifluoride diethyl etherate (80 µl, 0.70 mmol) was added to a stirred solution of 6-azido-6-deoxy-1,2,3,4-tetra-*O*-acetyl-D-galactopyranose **36** (α:β, 1:1) (52 mg, 0.139 mmol) in DCM (5 mL). The solution was stirred under argon at RT. After 20 min a solution of *p*-nitrophenol (19 mg, 0.14 mmol) in DCM (5 mL) was added to the reaction mixture and stirring under argon maintained. After 65 min, t.l.c. (DCM:ether, 60:1) indicated formation of two products (R_f 0.1, 0.4) with some remaining starting material (R_f 0.3). The DCM was removed *in vacuo*. The residue was diluted with chloroform (30 mL), washed with brine (3 x 30 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (DCM:ether, 60:1) to afford *p*-nitrophenyl 2,3,4-tri-*O*-acetyl-6-azido-6-deoxy-α-D-galactopyranoside **40** (9.7 mg, 14%) as a colourless oil; partial data $[\alpha]_D^{25}$ +18.3 (*c*, 0.5 in $CHCl_3$);ν_{max} (thin film) 3430 (br, OH), 2101 (sh, N_3), 1637 (st, C=O)cm^{-1} ; δ_H (500 MHz, $CDCl_3$) 2.05, 2.09, 2.21 (9H, 3 x s, 3 x CH_3), 3.13 (1H, dd, $J_{5,6}$ 4.2 Hz, $J_{6,6'}$ 13.1 Hz, H-6), 3.46 (1H, dd, $J_{5,6'}$ 8.2 Hz, H-6'), 4.18 (1H, dd, H-5), 5.34 (1H, dd, $J_{1,2}$ 3.7 Hz, $J_{2,3}$ 10.9 Hz, H-2), 5.49 (1H, d, $J_{3,4}$ 3.0 Hz, H-4), 5.57 (1H, dd, H-3), 5.94 (1H, d, H-1) 7.21 (2H, *J* 9.3 Hz,

2 x C<u>H</u>CHCNO$_2$), 8.26 (2H, 2 x C<u>H</u>CNO$_2$); δ_C (125.7 MHz, $CDCl_3$) 19.9, 20.0, 21.0 (3 x q, 3 x CH_3), 51.9 (t, C-6), 53.3 (d, C-2), 66.6 (d, C-3), 67.6 (d, C-4), 69.0 (d, C-5), 95.2 (d, C-1), 115.6, 115.7, 125.3 (3 x s, 3 x C=O), 116.9, 117.0 (2 x d, <u>C</u>HCHCNO$_2$), 126.5, 126.6 (2 x d, <u>C</u>HCNO$_2$), 140.8 (s, CNO$_2$), 167.2, 169.9, 170.2, 171.8 (4 x s, 3 x C=O, 1 x <u>C</u>CHCHCNO$_2$); *m/z* (CI+) 470 ($M + NH_4^+$, 10%).

o-Nitrophenyl β-D-galactopyranoside-6-phosphate 17

o-Nitrophenyl β-D-galactopyranoside **27** (903 mg, 3.0 mmol) was added to a mixture of trimethyl phosphate (7.5 mL, 64.8 mmol), water (0.05 mL, 3.0 mmol) and phosphorous oxychloride (0.84 mL, 9.0 mmol) at 0°C. The reaction mixture was stirred and after 2 h a change was observed from a white, cloudy suspension to a clear, yellow solution. After 3 h, t.l.c. (ethyl acetate:methanol, 4:1) indicated the formation of one product (R_f 0.2) and the absence of any starting material (R_f 0.3). Crushed ice (20 mL) was added and the reaction mixture neutralised with ammonia (5 mL of a 33% aqueous solution). The white crystalline solid thus formed was separated from the clear yellow solution by filtration, the filtrate concentrated *in vacuo* and co-evaporated with water (6 x 10 mL) to afford a white, crystalline solid. The residue was purified by flash column chromatography as follows; charcoal (10 g) and celite (10 g) were mixed together with hydrochloric acid (10 mL of a 1 M aqueous solution) and packed into a column. The white solid was dissolved in water (5 mL) and loaded onto the column. The column was eluted with water. Aliquots (1 mL) of each fraction were removed and tested for the presence of chloride ions by observing turbidity on addition of silver nitrate (1 mL of a 1 M aqueous solution). After elution with 1.75 L of water the presence of chloride ions were no longer detected. Further elution (water:pyridine, 2:1) yielded *o*-nitrophenyl β-D-galactopyranoside 6-phosphate **17** (701 mg, 62%) as a pale, yellow crystalline solid; m.p. 181.0-183.1°C (ethanol/ether) [Lit. ~180°C (ethanol/ether)];[27] $[\alpha]_D^{25}$ -31.1 (*c*, 0.2 in H_2O) [Lit. $[\alpha]_D^{20}$ -40 (*c*, 2 in H_2O)];[27] ν_{max} (KBr) 3400 (br, OH) 1527, 1355 (sh, C-NO_2), 1250 (sh, P=O)cm^{-1}; δ_H (400 MHz, D_2O) 1.17-1.29 (2H, m, 2 x C<u>H</u>NH$_3$), 1.52-1.57 (2H, d, *J* 12.6 Hz, 2 x C<u>H</u>CHCHCNH$_3$), 1.68-1.73 (4H, m, 4 x C<u>H</u>CHCNH$_3$), 1.87 (4H, br, 4 x C<u>H</u>CNH$_3$), 3.04 (2H, br, 2 x NH), 3.65-3.70 (2H, m, H-6, H-6'), 3.74-3.86 (3H, m, H-3, H-4, H-5), 3.91-3.92 (1H, m, H-1), 7.13-7.18

(1H, m, C<u>H</u>CHCNO$_2$), 7.33-7.42 (1H, m, C<u>H</u>CHCHCHCNO$_2$), 7.56-7.61 (1H, m, C<u>H</u>CHCHCNO$_2$), 7.83-7.86 (1H, m, C<u>H</u>CNO$_2$).

All applications, including U.S. Appln. No. 60/416,263, and publications are incorporated by reference herein.